(54) Title: METHODS AND COMPOSITIONS FOR ALTERING PHYSICAL CHARACTERISTICS OF FRUIT AND FRUIT PRODUCTS

(57) Abstract

Novel plants and methods for their preparation are provided which produce sucrase, particularly heterologous sucrase. The plants may have an increase in the soluble solids content of plant cells, particularly fruit cells as a result. DNA sequences encoding carbohydrate polymer synthesizing enzymes are identified, isolated and inserted into an expression cassette which may then be transformed into plant cells. The method finds particular use in conjunction with fruit-specific transcription for increasing production of carbohydrate polymers in fruit, particularly the apoplast.

METHODS AND COMPOSITIONS FOR ALTERING PHYSICAL CHARACTERISTICS OF FRUIT AND FRUIT PRODUCTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. application Serial No. 265,586 filed November 1, 1988, which is a continuation-in-part of U.S. application Serial No. 262,442, filed October 25, 1988, which is a continuation-in-part of U.S. application Serial No. 209,430, filed June 21, 1988, which applications are hereby incorporated herein by reference.

INTRODUCTION

Technical Field

This invention relates to the use of carbohydrate polymers to alter the physical characteristics of fruit and fruit products.

Background

Of significant interest to the fruit-processing industry are the physical characteristics of fruit such as the soluble solids composition of ripe fruit. Soluble solids affect storage, handling, commercial processing and cooking of the fruit. Of particular interest is optimization of soluble solids characteristics of succulent fruit with regard to properties such as consistency and/or viscosity.

In various applications, fruit having increased soluble solids, increased or decreased viscosity, or increased or decreased consistency, is desired. Increased soluble solids may be a function of "sink strength," the ability of the plant to translocate carbon to the fruit. Increased fruit sink

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activity is especially useful in fruit where high
soluble solids content is desirable, such as tomatoes
for processing into tomato-based products. Other
important fruit characteristics include properties

5   which relate generally to the cohesiveness of a
homogeneous mixture of the fruit and the viscosity of
the serum which remains after the pulp is removed
through centrifugation.

        Any modifications to fruit soluble solids,

10   should not however have adverse effects on other
desirable qualities or development of the plant or
fruit. This includes consideration of the physiolo-
gical consequences of the soluble solid, such as
effects of osmotic potential, and/or any subsequent by-

15   products on the plant, fruit and/or end-user,
particularly, when the fruit is used for food. It is
especially desirable that methods and compositions are
developed to modify soluble solids which are safe for
human consumption.

20   It is therefore of interest to obtain fruit
having an altered carbohydrate content which may thus
result in modified characteristics of the fruit or
fruit products. In particular, methods and
compositions for transforming a plant to produce fruit

25   having modified solids characteristic are desirable.
Furthermore, it is desirable not only to transform the
plant cell, but also to obtain the altered carbohydrate
composition at a particular stage in the growth of the
plant, particularly fruiting. In addition, it is

30   desirable that modification occur not only in a
particular tissue, such as fruit, but also in a
particular compartment of that tissue, such as the
apoplast. It is therefore important to be able to
express the carbohydrate polymer of interest using DNA

35   sequences which provide for the desired regulation of
expression in the plant host.

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Relevant Literature

cDNA clones from tomato displaying differen-
tial expression during fruit development have been iso-
lated and characterized (Mansson et al., Mol. Gen.
5   Genet. (1985) 200:356-361; Slater et al., Plant Mol.
Biol. (1985) 5:137-147). The studies have focused pri-
marily on mRNAs which accumulate during fruit ripening.
One of the proteins encoded by the ripening-specific
cDNAs has been identified as polygalacturonase (Slater
10   et al., Plant Mol. Biol. (1985) 5:137-147). A cDNA
clone which encodes tomato polygalacturonase has been
sequenced. Grierson et al., Nucleic Acids Research
(1986) 14:8395-8603. The concentration of polygalac-
turonase mRNA increases 2000-fold between the immature-
15   green and red-ripe stages of fruit development. This
suggests that expression of the enzyme is regulated by
the specific mRNA concentration which in turn is regu-
lated by an increase in transcription. DellaPenna et
al., Proc. Natl. Acad. Sci. USA (1986) 83:6420-6424.
20   In vitro synthesis and processing of tomato fruit
polygalacturanase has been described. DellaPenna et
al. Plant Physiol (1988) 86:1057-1063. Mature plastid
mRNA for psbA (one of the components of photosystem II)
reaches its highest level late in fruit development,
25   whereas after the onset of ripening, plastid mRNAs for
other components of photosystem I and II decline to
nondetectable levels in chromoplasts. Piechulla et
al., Plant Mol. Biol. (1986) 7:367-376.

30   SUMMARY OF THE INVENTION
Novel compositions and methods are provided
for modifying the soluble solids composition and
physical characteristics of fruit and fruit products.
The compositions include a novel DNA sequence encoding
35   Leuconostoc mesenteroides. The methods involve trans-
forming a plant cell of interest with an expression
cassette functional in a plant cell comprising a trans-

criptional and translational initiation regulatory
region, joined in reading frame 5' to a DNA sequence
encoding an enzyme capable of polymerizing a
carbohydrate comprising at least one hexose moiety to
5   form a carbohydrate polymer and translational and tran-
scriptional termination regions. To provide for
transport of the enzyme to a specific cellular
compartment, a DNA sequence encoding a transit peptide
may be included in the expression cassette. Expression
10   of the enzyme provides for an increase in the soluble
solids composition of the fruit as a result of
increased production of carbohydrate polymer. Of
particular interest is the replacement of fruit soluble
solids such as sucrose with carbohydrate polymers such
15   as dextran or levan, depending upon the desired
properties of the fruit or fruit products. Increased
concentration of carbohydrate polymer in fruit can be
used to alter not only the soluble solids composition
of the fruit, but also characteristics, for example
20   viscosity, of fruit products.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the integration of dextran-
sucrase in tomato fruit metabolism.
25   Figure 2 shows restriction maps of dextran-
sucrase (DS) clones pCGN1222, pCGN1223 and SALI partial
digests of pCGN1222 (-16, -18, -1, and -69).
pCGN1222-12 is a BamHI deletion of pCGN1222. Each
plasmid was transformed into E. coli DH1 and extracts
30   were assayed for DS activity (activity = cpm
14C-glucose incorporated into dextran): S = SalI;

P = PstI; X = XbaI; E = EcoRI; B = BamHI; K = KpnI.
Figure 3 shows the restriction map of L.
mesenteroides dextransucrase gene clone in the λ-fix
35   vector (ds-18) and its derivatives in pUC19. Arrows
indicate the direction of the lacZ promoter. The
expression of dextransucrase activity of each clone in

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E. coli is also shown. B, BamHI; E, EcoRI; H, HindIII;
K, KpnI; P, PstI; R, EcoRV; S, SalI; Sc, SacI; Sp,
SphI; X, XbaI.

5   Figure 4 shows the nucleotide sequence and
flanking regions of the dextransucrase gene from L.
mesenteroides. Numbering begins at the 5' end of the
sequence. The deduced amino acids are those matching
the four peptide sequence and the boxed amino acids are
those matching the four peptide sequences deduced from
10   amino acid sequence analysis. An arrow designates the
proposed cleavage site for signal sequence removal.
Putative promoter and ribosome binding site sequences
are underlined. Inverted repeated sequences are
underlined with broken arrows.

15   Figure 5 shows the amino acid analysis profile
of the dextransucrase protein. Plotted is an average
of three analyses. Cys and Trp percentages could not
be determined under HCl cleavage conditions. Error
bars indicate standard deviation. Protein sequence
20   matches located within the translated DNA sequences are
underlined.

Figure 6 shows a map of the 2All genomic
clone. The transcriptional start site, the
polyadenylation site, the start (ATG) and stop (TGA)
25   sites and the position of the intron are indicated.
The hatched region indicates the portion of the genomic
clone that was used to make the tagged 2All
constructions. The bottom portion shows the regions
used to construct the 2All cassettes including the
30   synthetic oligonucleotide used to insert restriction
sites and reconstruct the 3' end.

Figure 7 shows the 2All cassette pCGN1241
containing the ampicillin resistance gene.

Figure 8 shows a strategy for construction of
35   a PCR-generated dextransucrase construct.

Figure 9 shows a strategy of construction of
35S/dextransucrase and 2All dextransucrase binary

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vectors.

Figure 10 shows a strategy for construction of
a full length dextransucrase construct.

Figure 11 shows a strategy for cloning PG
leader in front of a dextransucrase gene.

Figure 12 is a Northern analysis of leaves
from plants transformed with B. subtilis levansucrase
(LVS) gene.

(A) Two transformants (4-2, 8-2) con-
tain transcripts that hybridize to the LVS probe, how-
ever only one transformant (8-2) is producing a full-
length transcript (~2 kb).

(B) mas 5'-LVS-mas 3' construct in
pCGN1205.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention,
novel DNA sequences, DNA constructs, methods and
compositions are provided which permit modification of
the soluble solids composition of fruit by means of an
increased synthesis of carbohydrate polymer from
carbohydrates such as sucrose which comprise at least
one hexose moiety. Plant cells are transformed with an
expression cassette comprising a DNA sequence encoding
a an enzyme such as a sucrase capable of polymerizing a
carbohydrate such as sucrose to form a carbohydrate
polymer. Timing of expression and/or tissue
specificity, may be provided by the use of trans-
criptional regulatory regions having the desired
expression specificity. The DNA sequence may also
include a DNA sequence encoding a transit peptide
recognized by the plant host to provide for targeting
to a specific compartment within a tissue of interest,
such as fruit apoplast.

Modification of the soluble solids composition
of a fruit offers potential means to alter specific
properties of the fruit. By "modification of the

soluble solids composition of fruit" is intended the
replacement of sucrose, glucose and fructose or other
soluble sugars with carbohydrate polymers. Of special
interest are carbohydrate polymers which are not
5   readily degraded. Although degradation of the polymer
may be useful to achieve desired properties in some
instances, most preferred is an investment in solids
which will not be withdrawn later by degradation or
other means. A related concern is the potential for an
10   undesired accumulation of hexose monomers which may
cause stress to the cell. Thus, polymers which are
"unfamiliar" carbohydrate polymers, i.e., polymers
which are not naturally found in the particular plant
host of interest, may have special use because the
15   plant is unlikely to possess an enzyme capable of
degrading the unfamiliar polymer.

In addition to the effects of a modified
carbohydrate profile from replacement of sucrose
carbons with carbohydrate polymer carbons, the soluble
20   solids of the fruit may be increased by the stimulation
of added carbon flow to the fruit. If the draw of
sucrose to carbohydrate polymer strengthens carbon-sink
activity in the fruit, a corresponding increase in
soluble solids may be achieved.

25   Considerations for use of a specific sucrase
in fruit for the conversion of sucrose to a
carbohydrate polymer include pH optimum of the enzyme,
the availability of substrate and co-factors required
by the enzyme. The sucrase enzyme of interest should
30   have kinetic parameters compatible with the biochemical
found in the host plant cell. For example, sucrase may
have to compete for substrate with other enzymes, such
as invertase. Analysis of the K_m and specific activity
of the enzymes in question should be considered in
35   determining the suitability of a sucrase for polymer
production in a given host plant. Furthermore, in some
cases, it may be desirable to target the enzyme to a

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specific location. For example, in tomato it is
desired to target the sucrase to the apoplast. The
enzyme would thus need to be one which can function
under conditions present in the apoplast.

5   For purposes of this invention, it is
preferable to replace the native soluble solids with
carbohydrate polymers which have, for example,
different viscosity and/or consistency coefficients
from the soluble solids of the fruit host. Replacement

10   of tomato solids with levan, a viscous carbohydrate in
pure solution, reduces gross viscosity and serum
viscosity of tomato paste under simulated processing
conditions. Replacement of tomato solids with dextran
increases the gross viscosity of tomato paste. Thus

15   the use of levansucrase and dextransucrase constructs,
respectively, are of particular interest.

         To provide for an increased expression of
sucrase in a plant, a plant cell is transformed with an
expression cassette which includes in the 5'-3' direc-

20   tion of transcription, a transcriptional and trans-
lational initiation region functional in a plant cell;
a structural gene encoding a sucrase, preferably in-
cluding a sequence encoding a transit peptide in
reading frame at the 5'-terminus, where the transit

25   peptide directs transfer of the sucrase to the apo-
plast; and a transcriptional and translational
termination regulatory region. By sucrase is intended
an enzyme capable of converting sucrose to a
carbohydrate polymer. The initiation and termination

30   regulatory regions are functional in the host plant
cell and may be either homologous or heterologous to
the host plant.

         The sucrase gene may be derived from prokaryo-
tic sources, for example, genes for dextransucrase, and

35   other glycosyltransferases, which polymerize the
glucose moiety of sucrose to form dextran, may be
obtained from various species of Leuconostoc and

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Streptococcus, including the soil bacterium L.
mesenteroides. Genes for levansucrase, which catalyzes
the conversion of sucrose to levan, a fructose polymer
and free glucose, may be obtained from a variety of
5   species of bacteria, for example, Aerobacter levanicum,
Streptococcus salivarius and Bacillus subtilis.
Preferentially the dextransucrase gene is derived from
Leuconostoc mesenteroides and the levansucrase is
derived from Bacillus subtilis. The DNA sequence
10   encoding L. mesenteroides dextransucrase is provided.
Dextransucrase derived from L. mesenteroides strain B-
512F catalyzes the polymerization of branched dextrin
(95% α1->6 linkage and 5%-1->3 branched linkages) from
sucrose. In contrast to Streptococcus strains, which
15   typically produce several distinct glucosyltransfer-
ases, L. Mesenteroides B-512F produces only one glyco-
syltransferase, and at high levels. Comparison of the
deduced amino acid sequence of dextransucrase with
other sequenced proteins (data base) revealed partial
20   homologies with two other glucoslytransferase
proteins: gtfB from S. sobrinus (41.5%) and gtfB from
S. mutants (41.7%).

Eukaryotic sources, including plant sources,
such as Jerusalem artichoke and some grasses, may also
25   find application. Examples of plant enzymes which also
may be used include those involved in the synthesis of
fructans from sucrose from a variety of sources (see,
for example, Bhatia et al., Phytochem. (1979) 18:923-
927; Edelman et al., New Phytol. (1968) 67:517-553;
30   Henby et al., Phytochem. (1980) 19:1017-1020;
Satyanarayana, Ind. J. Biochem. Biophys. (1976) 13:261-
266; Shiomi, Agric. Biol. Chem. (1980) 44:603-614;
Shiomi, Carbohydrate Research (1981) 96:281-292;
Shiomi, Carbohydrate Research (1982) 99:157-169; and
35   Singh, Phytochem. (1971) 10:2037-2039).

The structural gene for the sucrase may be obtained in a variety of ways. The gene may be synthesized in whole or in part, particularly where it is desirable to provide plant preferred codons. Thus, all

5   or a portion of the open reading frame may be synthesized using codons preferred by the plant host. Plant preferred codons may be determined from the codons of highest frequency in the proteins expressed in the largest amount in the particular plant species of

10   interest.

Methods for synthesizing sequences and bringing the sequences together are well established in the literature. Where a portion of the open reading frame is synthesized, and a portion is derived from natural

15   sources, the synthesized portion may serve as a bridge between two naturally occurring portions, or may provide a 3'-terminus or a 5'-terminus. Particularly where the signal sequence and the open reading frame encoding the sucrase are derived from different genes,

20   synthetic adapters commonly will be employed. In other instances, polylinkers may be employed, where the various fragments may be inserted at different restriction sites or substituted for a sequence in the polylinker.

The sucrase structural gene may be derived

25   from cDNA, from chromosomal DNA or may be synthesized in whole or in part. For the most part, some or all of the sucrase structural gene will be from a natural source or genes substantially homologous to such sequences. In some situations it may be desirable to

30   modify all or a portion of the codons, for example to enhance expression, by employing host-preferred codons. Methods for identifying sequences of interest have found extensive exemplification in the literature, although in individual situations, different degrees of

35   difficulty may be encountered. Various techniques include the use of probes where genomic or cDNA libraries may be searched for complementary sequen-

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ces. Where the structural gene to be inserted is de-
rived from prokaryotic cells, it is desirable to
minimize the 3' non-coding region of the prokaryotic
gene. The substantial absence of this untranslated

5   region can have a positive effect on the transcription,
stability, and/or translation of the mRNA in the plant
cells.

When the structural gene is other than a plant
gene, in order to have expression of the gene in a plant

10   cell transcriptional and translational initiation regu-
latory regions functional in a plant cell must be pro-
vided. Promoters and translation initiation signals
functional in plant cells include those from genes
which are present in the plant host or other plant spe-

15   cies, for example the ribulose bisphosphate carboxylase
small subunit transcriptional initiation region, for
example from tobacco; those present in viruses such as
the cauliflower mosaic virus (CaMV), for example the
35S transcriptional initiation region; and those asso-

20   ciated with T-DNA such as the opine synthase transcrip-
tional initiation regions, for example, octopine, man-
nopine, agropine, etc. Of particular interest is a
transcriptional initiation region in a construct
comprising two 35S promoters in tandem; fruit specific

25   promoters, e.g. 2A11 (see co-pending application).
The regulatory regions may be homologous
(derived from the original host) or heterologous
(derived from a foreign source, or synthetic DNA
sequence) to the plant host. In order to join the

30   promoter(s) to the structural gene, the non-coding 5'
region upstream from the structural gene may be removed
by endonuclease restriction. Alternatively, where a
convenient restriction site is present near the 5'
terminus of the structural gene, the structural gene

35   may be restricted and an adapter employed for linking
the structural gene to a promoter region, where the
adaptor provides for lost nucleotides of the structural

gene.

Transport of the sucrase into a particular
cellular compartment may be accomplished by the use of
a transit peptide to target a cellular compartment of
5   interest, such as the apoplast. The transit peptide is
inserted following the promoter sequence(s). The
transit peptide and processing signal may be derived
from any plant protein which is expressed in the
cytoplasm and translocated to the cellular compartment
10   of interest. The transit peptide can be identified by
comparing the messenger RNA from the particular
polypeptide with the mature product. The amino acid
sequence absent from the mature protein and coded for
by the messenger beginning at the initiation codon,
15   usually a methionine, will normally be the transit se-
quence. Fragments from the natural transit sequence
which retain their transport activity can also be used.
The transit peptide is the sequence capable of direc-
ting a peptide joined to the transit peptide to the
20   cellular compartment of interest and may be the whole
wild-type transit peptide, a functional fragment
thereof, or a functional mutant thereof. The full
natural sequence is also included.

For the most part, the transit peptides from
25   one plant are generally recognized by other plants.
Thus the transit peptide may be native to or heterolo-
gous to the ultimate host in which the chimeric gene
comprising the structural gene and transit peptide is
introduced. Transit peptides may come from tomato or
30   other fruits or vegetables. The transit peptide will
usually have at least about 20 amino acids and not more
than about 100 amino acids. Transit peptides of
particular interest include leader sequences from genes
encoding apoplast proteins produced in the cytoplasm,
35   then translocated to the apoplast. Such proteins

include polygalacturonase (see Sheehy et al., Mol. Gen. Genet. (1987) 208:30-36), a cell wall enzyme implicated in the softening of fruit during ripening.

The termination region may be derived from the 3'-region of the gene from which the initiation region was obtained or from a different gene. The termination region may be derived from a plant gene, particularly the tobacco ribulose biphosphate carboxylase small sub-unit termination region; a gene associated with the Ti-plasmid such as the octopine synthase termination region; or the tml termination region.

In developing the expression cassette, the various fragments comprising the regulatory regions and open reading frame may be subjected to different processing conditions, such as ligation, restriction enzyme digestion, resection, in vitro mutagenesis, primer repair, use of linkers and adapters, and the like. Thus, nucleotide transitions, transversions, inser-tions, deletions, or the like, may be performed on the DNA which is employed in the regulatory regions and/or open reading frame. The expression cassette thus may be wholly or partially derived from natural sources, and either wholly or partially derived from sources homologous to the host cell, or heterologous to the host cell. Furthermore, the various DNA constructs (DNA sequences, vectors, plasmids, expression cassettes) of the invention are isolated and/or purified, or synthesized and thus are not "naturally occurring".

During the construction of the expression cas-sette, the various fragments of the DNA will usually be cloned in an appropriate cloning vector, which allows for amplification of the DNA, modification of the DNA or manipulation by joining or removing of sequences, linkers, or the like. Normally, the vectors will be capable of replication in at least a relatively high copy number in E. coli.

A number of vectors are readily available for cloning, including such vectors as pBR322, pUC series, M13 series, etc. The cloning vector will have one or more markers which provide for selection for transfor-
5   mants. The markers will normally provide for resistance to cytotoxic agents such as antibiotics, heavy metals, toxins, or the like. By appropriate restriction of the vector and cassette, and as appropriate, modification of the ends, by chewing back or filling in
10 overhangs, to provide for blunt ends, by addition of linkers, by tailing, complementary ends can be provided for ligation and joining of the vector to the expression cassette or component thereof.

After each manipulation of the DNA in the de-
15 velopment of the cassette, the plasmid will be cloned and isolated and, as required, the particular cassette component analyzed as to its sequence to ensure that the proper sequence has been obtained. Depending upon the nature of the manipulation, the desired sequence
20 may be excised from the plasmid and introduced into a different vector or the plasmid may be restricted and the expression cassette component manipulated, as appropriate. The manner of transformation of E. coli with the various DNA constructs (plasmids and viruses)
25 for cloning is not critical to this invention. Conjugation, transduction, transfection or transformation, for example, calcium chloride or phosphate mediated transformation, may be employed.

Depending upon the manner of introduction of
30 the expression construct into the plant, other DNA sequences may be required. Commonly, the expression cassette will be joined to a replication system functional in prokaryotes, particularly E. coli, so as to allow for cloning of the expression cassette for isola-
35 tion, sequencing, analysis, and the like. Included with the replication system will usually be one or more markers which may allow for selection in the host, the

markers usually involving biocide resistance, for exam-
ple antibiotic resistance; heavy metal resistance;
toxin resistance; complementation, providing photo-
trophy to an auxotrophic host; immunity; etc. Where
5   the DNA will be microinjected into the host cell, a
marker will usually be desirable which allows for sel-
ection of those cells in which the injected DNA has
become integrated and functional. Thus, markers will
be selected which can be detected in a plant host.

10   Alternatively, one may use agrobacteria as a
vector for introducing DNA into a plant cell host. A
first host vector is transformed with an extrachro-
mosomal element or plasmid capable of maintenance in
said first host, where said extrachromosomal element is
15   characterized as being self-transmissable or capable of
mobilization with a second helper extrachromosomal
element; being capable of conjugal transfer to agrobac-
teria; containing predetermined DNA segments comprising
foreign nucleic acid sequences, such as foreign DNA,
20   encoding a peptide or other genetic information of
interest capable of replication, transcription and usu-
ally translation in a host cell; and at least one DNA
segment homologous with target DNA in agrobacteria, in
said agrobacteria, either chromosomal or extrachro-
25   mosomal; and optionally a marker for selection.
Generally, the extrachromosonal elements comprise a
vector having a replicon or replication system having a
narrow host range. That is, the extrachromosomal
elements are capable of maintenance and replication in
30   a first host which is capable of conjugation with
agrobacteria having the target DNA, but the vector is
incapable of maintenance in the agrobacteria so that
the agrobacteria is rapidly cured. In this way, in the
absence of integration of at least a portion of the
35   extrachromosomal composition, the extrachromosomal DNA
will be lost in the agrobacteria. Thus, the DNA of the
extrachromosomal element is rescued by integration with

a stable genetic element of the agrobacteria.

The replicon may come from a plasmid, virus, or other source which allows for stable maintenance in the first host. The first host will normally be a pro-karyote, particularly E. coli. By employing a vector capable of replication in E. coli and other enteric hosts (narrow range), the vector will not be replicated and maintained in non-enteric Gram negative bacteria. The extrachromosomal element may therefore be a plasmid or virus (phage) or other DNA, which provides the requisite properties.

The extrachromosomal element may also have a mobilization locus, and be self-transmissable, or capa-ble of conjugal transfer by trans-complementation with a helper plasmid having the necessary genes for trans-fer, e.g., tra. Conveniently, the extrachromosomal DNA and the helper plasmid will be initially employed in different cells, employing tripartite mating, or a variation thereof, rather than dual mating, so that in vitro introduction of both the extrachromosomal DNA and the helper plasmid into the first host cell prior to conjugal transfer is not required. The helper plasmid will have the necessary function for conjugal mobiliza-bility (transfer), as well as a functioning replication system. Another characteristic is the presence of a DNA sequence in the extrachromosomal element of at least about 50bp which is at least substantially homo-logous with DNA present in the agrobacteria to provide for efficient integration.

The homologous DNA in the agrobacteria which serves as the recombination locus may be chromosomal or extra chromosomal. Of particular interest is the T-DNA region of the Ti plasmid, the region of the Ti plasmid between the left and right borders. The region of homology may include regions encoding proteins, regu-latory regions, or non-encoding regions and need not have perfect homology. By having regions of at least

substantial homology with the target DNA on both sides
of the sucrase expression cassette and in close
proximity to the termini of the cassette DNA, the
cassette may be inserted into the target host DNA with
5   a minimum amount of the remaining DNA of the
extrachromosomal element. Where it is desired to
obtain normal plant cells and plants following
transformation, the T-DNA sequences used to provide
homology will not include the tumor genes, or the tumor
10   genes will have been rendered inactive using means well
known to those skilled in the art.

     The region of homology will be of a sufficient
size to provide for integration, and generally will be
about 3000bp, more usually at least about 1000bp,
15   conveniently 500bp, and may be 100bp or 50bp as to each
of the fragments. The region of homology is generally
present in two segments flanking the nucleic acid
sequence of interest, the total number of bases of the
segments generally not exceeding about 2000bp. The two
20   segments generally not exceeding about 2000bp. The two
segments may be the same or different, both as to size
and sequence, or may be a wild type or mutant T-DNA
sequence, interrupted by the intervening sequences;
alternatively, one T-DNA sequence will suffice as
25   described by Comai et al., Plasmid (1983) 10:21-30.

     The use of T-DNA for transformation of plant
cells has received extensive study and is amply de-
scribed in EPA Serial No. 120,516, Hoekema, In: The
Binary Plant Vector System, Offsetdrukkerij Kanters
30   B.V., Alblasserdam, 1985, Chapter V, Knauf, et al.,
"Genetic Analysis of Host Range Expression by Agrobac-
terium", In: Molecular Genetics of the Bacteria-Plant
Interaction, Puhler, A. ed.,Springer-Verlag, NY, 1983,
p. 245, and An et al., EMBO J. (1985) 4:277-284.
35   Conveniently, explants, cotyledons, or other
plant tissue may be cultivated with A. tumefaciens or
A. rhizogenes to allow for transfer of the expression

construct to the plant cells, the plant cells dispersed
in an appropriate selective medium for selection, grown
to callus, shoots grown and plantlets regenerated from
the callus by growing in rooting medium. The agrobac-
5　terium host will contain a plasmid having the vir genes
necessary for transfer of the T-DNA to the plant cells
and may or may not have T-DNA. If the expression
construct is to be inserted into the host cell by
injection or electroporation, disarmed Ti-plasmids
10　(lacking the tumor genes, particularly the T-DNA
region) may be used.

　　　　Various techniques exist for determining whe-
ther the desired DNA sequences present in the plant
cell are integrated into the genome and are being tran-
15　scribed. Techniques such as the Northern blot can be
employed for detecting messenger RNA which codes for
the sucrase. In addition, the presence of expression
can be detected in a variety of ways, such as assaying
for enzyme activity or immunoassay for the protein
20　product. A desired phenotype in this case is the
presence of increased solids as polymers in the plant
fruit, especially the presence of dextran or levan.

　　　　The cells which have been transformed may be
grown into plants in accordance with conventional ways.
25　See, for example, McCormick et al., Plant Cell Reports
(1986) 5:81-84. These plants may then be grown, and
either pollinated with the same transformed strain or
different strains, identifying the resulting hybrid
having the desired phenotypic characteristic. Two or
30　more generations may be grown to ensure that the sub-
ject phenotypic characteristic is stably maintained and
inherited and then seeds harvested for use to provide
fruits with the new phenotypic property, particularly
increased solids due to production of carbohydrate
35　polymers, especially levan and dextran.

　　　　Modification of the carbohydrate profile of
fruit may be used to alter the characteristics of fruit

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products destined for human consumption. Thus, the
safety of any food additive is an important
consideration. Dextran is used clinically as a blood
plasma extender. It formerly had U.S. "G.R.A.S."
5   (generally recognized as safe) status as a food
additive, but was dropped from the list solely due to
disuse. One mole of glucose or fructose is released by
the respective reactions of dextransucrase and
levansucrase for each mole of sucrose consumed. Thus,
10   even massive sucrose conversion should not "starve" the
other metabolic needs of the fruit. Since the osmotic
potential of one mole of sucrose is relatively
equivalent to one mole of glucose or fructose monomer,
the plant additionally will not be stressed and yield
15   affected.

          The following examples are offered by way of
illustration and not by limitation.

20   EXPERIMENTAL

          E. Coli 71-1.8; pCGN783 was deposited with the
American Type Culture Collection (ATCC), 12301 Parklawn
Drive, Rockville, Maryland, 20852, on December 23, 1988
and given Accession No. 67868.

25

                         Example 1
              Effects of Carbohydrate Polymers
                 on Viscosity of Tomato Paste

Effects on Viscosity
30   Polymers in water were added to tomato paste,
which was then evaporated to achieve the desired level
of total solids for each measurement. In all of these
experiments, the polymer replaces part of the tomato
solids. As a result, no change in the viscosity of the
35   paste would be expected if the viscosity of the added
polymer is equal to the average viscosity of the tomato
solids that were replaced.

A.   <u>Levan</u>

       Modeling studies were done with levan from <u>Aerobacter</u> <u>levanicum</u>. This polymer is similar in structure to the levan produced by <u>Streptococcus</u> <u>salivarius</u>. Although levan is very viscous in pure solution, the polymer appears to break down under simulated tomato processing conditions. Thus, levan caused reductions in the gross viscosity and the serum viscosity of the tomato paste (Table 1).

Table 1

Effect Of Levan On
Tomato Paste Viscosity

Hot break tomato juice	Bostwick (cm)	Ostwald (centisoles)	
control		4.5	597
+0.75% levan	5.8	198	

Bostwick consistency was measured at 10% total solids. Ostwald viscosity was measured on clear serum from paste at 6% total solids.

B.   <u>Dextran</u>

       Preliminary results from addition of dextran to tomato paste suggested that a dextran obtained from the <u>Leuconostic</u> <u>mesertoroides</u> B-512F increased Ostwald viscosity in both hot break paste and cold break paste (Table 2).

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Table 2

Effect Of Dextran On
Tomato Paste Viscosity

Cold break tomato paste	Bostwick (cm)	Ostwald (centisoles)
control	7.5	152
+0.5% dextran	9.5	297

Hot break tomato paste	Bostwick (cm)	Ostwald (centisoles)
control	6.2	271
+0.5% dextran	6.5	430

Table 2 (cont'd)

Bostwick consistency was measured at 13% total solids
(cold break paste) or at 10.8& total solids (hot break
paste). Ostwald viscosity was measured on clear serum
from paste at 6% solids.

C.   Inulin

        Inulin is a storage polymer produced by a
number of higher plants which are grown for human
consumption (e.g. Jerusalem artichoke and dandelion).
No consistent effect on viscosity was observed when an
inulin suspension was added to tomato paste at room
temperature. The inulin used did not dissolve
completely, however, unless heated to 80°C. Heating
the inulin together with the tomato paste provided a
more accurate model of how the inulin would be
dispersed through the paste during processing. The
temperature of the paste was then lowered for viscosity
measurements. The result of this treatment was an
increase in gross viscosity of the inulin-containing
paste (Table 3).

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Table 3

Effect Of Inulin On
Tomato Paste Viscosity

(a) Inulin suspension added to cold break tomato paste.

Trial		Bostwick (cm)	Ostwald (centisoles)
Trial	control	10.0	83
	+0.5% inulin	8.9	63
Trial II	control	11.0	71
	+0.5% inulin	12.9	65

(b) Inulin suspension added to hot break tomato paste.

Trial		Bostwick (cm)	Ostwald (centisoles)
Trial I	control	6.0	270
	+0.5% inulin	7.0	271
Trial II	control	6.5	271
	+0.5% inulin	5.7	225

(c) Inulin heated together with cold break tomato paste.

Trial		Bostwick (cm)	Ostwald centisoles
Trial I	control	11.0	71
Trial II	+0.5% inulin	6.5	79

Dextransucrase Enzymology

Dextransucrase activity was assayed under conditions chosen to approximate the environment of the tomato fruit apoplast as follows: 10 mM sucrose, 50 mM

glucose, 50 mM fructose, 3 mM $CaCl_2$, 50 mM sodium
acetate pH 4.2, 0.02% NaN_3, 0.1 mg/ml Tween 80 and
25°C. (Daman et al. Plant Physiol. (1988) 87:731-
736) Dextran polymer was isolated by ethanol
5   precipitation, lyophilized and redissolved in 0.02%
NaN_3. Dextran was assayed by the phenol-sulfuric acid
method using glucose as the standard. Under these
conditions, dextransucrase converted most of the
sucrose into dextran polymers. The results were as
10 shown in Table 4. Gel filtration and dextranase
digestion demonstrated that the dextran synthesized in
vitro had a structure indistinguishable from that of
native dextrans. Only small amounts of the acceptor
reaction products leucrose and isomaltose were
15 produced. Thus, glucose and fructose were not readily
used as acceptors and, at the concentrations found in
tomato, they did not prevent the synthesis of high
molecular weight dextran polymers.

20

Table 4

Dextransucrase: Reaction in vitro

	EtOH-precipitable carbohydrate formed within 72 hours	
reaction mixture	mg	% yield
complete (14 U/ml enzyme)	18	74
-minus enzyme control	none	0
-boiled enzyme control	none	0
-minus sucrose control	none	0

30

High molecular weight dextran polymer was also
produced when dextransucrase and sucrose were added to
35 serum from ripe tomato fruits (Table 5). This
indicates that the tomato does not contain significant
concentrations of any unknown substances that prevent

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dextransucrase from producing high molecular weight dextran (e.g., inhibitors or acceptors).

Table 5

Dextran Synthesis in Tomato Fruit Serum[1]

Reaction Mixture		High molecular weight carbohydrate (μg)
a	ripe fruit serum	0
b	serum+sucrose	74
c	serum+dextransucrase	40
d	serum+sucrose+dextransucrase	308
e	reaction mixture d + dextranase	80

[1]Sephacryl S-1000 gel filtration chromatography was performed on the reaction mixtures above. High molecular weight carbohydrate which eluted at the void volume had a molecular mass of >100,000 kDa. The 108 μg of high molecular weight carbohydrate in the original serum was substrated from the carbohydrate content of all reaction mixtures. High molecular weight carbohydrate was synthesized when sucrose and dextransucrase were added together (reaction mixture d). The newly synthesized dextran was hydrolyzed to low molecular weight products by treatment with dextranase enzyme (reaction mixture e).

In order to function in the tomato fruit apoplast, dextransucrase must compete for substrate with invertase (see Figure 1). The K_m for sucrose and the specific activity of these two enzymes were relatively close as shown in Table 6, below.

Table 6

Integration of Dextransucrase in Tomato Fruit Metabolism

	Km for sucrose (mM)	Specific activity (units/mg protein)
Tomato fruit invertase	6	320
Dextransucrase	14	170

25

Thus, dextransucrase can compete successfully for
substrate and dextran polymers can be produced in the
engineered fruits. At the other extreme, even massive
dextran production should not "starve" the other
metabolic needs of the fruit because one mole of
fructose is released for every mole of sucrose consumed
in dextran production.

Example 2
Isolation and Characterization
of Dextransucrase Clones

Construction of L. mesenteroides DNA Library and
Screening for Clones Expressing the Dextransucrase Gene

A Sau3A/Lambda-Fix (Stratagene) library was
constructed, following the procedures supplied by the
manufacturer, from L. mesenteroides DNA isolated from a
late exponential phase culture of L. mesenteroides
grown in GYC medium at 30°C. GYC medium has the follow-
ing formulation: 10 g/l potassium phosphate (pH 6.7);
2 g/l yeast extract (Difco); 5 g/l casein hydrolysate
(Gibco); 20 g/l glucose; and 5 ml/l salts solution (40 g
$MgSO_4.H_2O$, 2 g NaCl, 2 g $FeSO_4.7H_2O$, and 2 g $MnSO_4.H_2O$
per liter of water). DNA was partially digested with
Sau3A to generate a random population of fragments
averaging approximately 15-20 kb in size. Lambda-Fix
is an EMBL vector modified so that the phage arms can-
not ligate to themselves or to the stuffer fragment.
In addition, the Sau3A partially-digested DNA is modi-
fied so that it cannot form multimers, eliminating the
need to size fractionate the insert DNA. Sau3A par-
tially-digested L. mesenteroides DNA was ligated into
Lambda-Fix and packaged in vitro into viable phage
particles. The Sau3A/Lambda-Fix library consists of
approximately 100 genomes worth of L. mesenteroides DNA
consisting of 10^5 plaque-forming units.

Recombinant phage (5x10^3) were screened for
dextransucrase expression by the method of Gilpin et
al. (Infect. Imm. (1985) 49:414) by plating with E.
coli in Luria top agar (10 g/l tryptone, 5 g/l yeast
extract, 10 g/l NaCl, 0.8% agar) over M-9 minimal salts
agar (30 g/l 30g/l KH$_2$PO$_4$; 53g/l Na$_2$HPO$_4$; 5g/l NaCl;
10g/l NH$_4$Cl; 2g/l glucose; 1mM MgSO$_4$; 0.1mM CaCl$_2$),
containing 0.4% sucrose; 0.1mg/ml dextran T-10; 1µg/ml
thiamine; 8µg/ml leucine: 8µg/ml threonine, pH 7.5, and
incubating at 3 °C. The E. coli (strain C600) cannot
utilize sucrose as a carbon source, therefore only weak
growth of bacteria occurs in the top agar generating
very small plaques. When the dextransucrase gene is
present and expressed, dextran is produced and appears
as a white halo around the plaque. Growth of E. coli
is also stimulated around the plaque, presumably
feeding off of the monosaccharides being released as
dextrans are formed. Approximately 1% of the phage
screened produced white halos of polymer around the
plaques.

Dextran-producing plaques were isolated and
partially purified. The clones were further analyzed
by assaying for the incorporation of
^{14}C into polysaccharide polymer from sucrose labeled
specifically in the glucose or fructose moiety.
Polymer was picked from each of the putative
dextransucrase clones growing in Luria top agar over
sucrose minimal agar as described above and suspended
in 100µl of DS buffer (200mM NaOAc, pH5.2, 0.1g/l
Dextran T-9, 1.0g/l Tween 80, 1.0mM CaCl$_2$,
0.04% NaN$_3$). Half of the suspension (50µl) was then
transferred to a reaction tube containing 0.1µCi of
dried sucrose [glucose-^{14}C] with 50µl DS substrate (DS
buffer, 6.8g/l sucrose) and the other half to a
reaction tube containing sucrose [fructose-^{14}C] with
50µl DS substrate. The reactions were then incubated
at 30°C for approximately 16 hrs. An aliquot of each

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reaction (10-30µl) was then spotted onto Whatman No.1
filter paper. The filters were washed in three changes
of methanol (10ml/filter/change) on a table top shaker
for 10 minutes/change, then air dried and counted in
5   5ml scintillation fluid in a Beckman Scintillation
Counter. Germaine et al, J. Dent. Res. (1974) 53:
1355-1360. All of the clones incorporated
sucrose-derived ^{14}C-fructose and not ^{14}C-glucose into
polymer indicating that the clones were expressing
10 levansucrase rather than dextransucrase. In addition,
reactivation of the enzyme produced after SDS-PAGE and
PAS staining for carbohydrates (Miller and Robyt, Anal.
Biochem. (1986) 156:357-363) revealed a protein with
the mobility expected-for levansucrase (116 kd).
15   Rescreening of the Lambda-Fix library was
performed as described above except that the medium was
first buffered to pH 6. The pH optimum of dextran-
sucrase is pH 5.2. LB media is approximately pH 7.5
and increases to approximately pH 8 as the E. coli
20 grow. At pH 8, dextransucrase retains only 10% of
maximal activity, but this value is 80% at pH 6.0.
Twenty clones producing halos were picked and assayed
by the ^{14}C-labeling assay described above. One clone,
DS-18, incorporated sucrose derived ^{14}C-glucose and was
25 purified.

Example 3
Analysis of cDNA Clones
Determination of dextransucrase activity
30   DNA was isolated the DS-18 lambda clone and a
partial restriction map was produced. A fragment
containing the dextransucrase gene was subcloned as an
XbaI fragment from DS-18 into pUC19 in both
orientations (pCGN1222, pCGN1223). The plasmids were
35 used to transform a DNA stabilizing strain of E. coli
(DH1, recA). Maniatis et al., Molecular Cloning: A
Laboratory Manual (Cold Springs Harbor, 1982).

Extracts were then prepared and assayed for DS activity
by the sucrose [glucose-^{14}C] incorporation assay
described above. Only one orientation of the insert
(pCGN1222) resulted in expression of DS activity.

5   Plasmids containing SalI partial deletions of pCGN1222
in DH1 were plated on SYC media (same as GYC media
except sucrose is substituted for glucose) and grown at
30°C until colonies showed the large viscous colonies
characteristics of dextran production at approximately

10   nine days. The clones were further analyzed for DS
activity by the sucrose [glucose-^{14}C] incorporation
assay described above. Those colonies producing
dextran also incorporated ^{14}C-glucose while colonies
not producing dextran did not incorporate

15   ^{14}C-glucose. Due to the orientation-dependent
expression of DS described above, the 5' end of the DS
gene was proposed to be at the left end of the pCGN1222
insert as shown in Figure 2.

Additional information was obtained by
20   deletion of the BamHI fragment from pCGN1222 to form
pCGN1222-12 (Fig. 2). The resulting plasmid retained
the ability to incorporate ^{14}C-glucose from sucrose
[glucose-^{14}C], although the activity appeared
reduced. The remaining 5' 6.8kb fragment therefore

25   presumably contains the majority of the DS gene, with
perhaps some portion of the gene located beyond the
BamHI site. Deletions were made to the pCGN1222
derivatives shown in Figure 2 to form the corresponding
plasmids shown in Figure 3 using Promega's Erase-a-Base

30   system. Progressive unidirectional deletions were made
using exonuclease III which specifically digests DNA
from 5' protruding or blunt ends while leaving 4 base
3' protruding ends intact. For example, plasmid
pCGN1222-12 was cut with XbaI to generate a 5'

35   protruding end and deletions were made approximately
3kb into the insert. The plasmid was also cut with
SphI to generate a 3' protruding end thus protecting

the vector from digestion. Deletions were then trans-
formed into E. coli (DH1), plated onto SYC media, and
screened for DS activity by the sucrose [glucose-^{14}C]
incorporation method described above. Plasmid DNA was
5   isolated and restriction mapped from various colonies,
both positive and negative for DS activity. The 5'
boundary of the DS coding region was determined by
comparing the restriction maps and DNA sequences of two
clones, of pCGN2023, clones 5-1 and 5-7. Clone 5-1
10   does not incorporate ^{14}C-glucose. The 5' end of the
insert maps 161bp 5' to the first PstI site and does
not contain the first HindIII site. Clone 5-7 does
incorporate ^{14}C-glucose and the 5' end of the insert
maps 265bp 5' to the first PstI site and contains the
15   first HindIII site (Fig. 3). Clone 5-1 does not
contain the putative ATG initiation codon. Clone 5-7
contains an ATG (Met) codon which begins a 4.78kb open
reading frame, and therefore is proposed to be the
start site for DS translation. Clone 5-7 represents
20   the shortest active fragment obtained.

DNA Sequencing

        The DNA sequence of the dextransucrase (DS)
gene was determined by the dideoxy-chain termination
method of Sanger et al. (1977) using M13 single-
25   stranded templates or double-stranded DNA (Chen and
Seeburg) and the modified bacteriophage T7 polymerase
(Sequences) (Tabor and Richardson). Fragments were
subcloned into M13mp18 (Yannich-Perren et al., Gene
(1985) 33:103-119) or a bluescribed M13+ chlor-
30   amphenicol derivative (Stratagene, San Diego, CA) and
nested deletions were created using the Erase-a-Base
System (Promega Biotec). Synthetic oligonucleotide
primers were synthesized on an Applied Biosystems model
380A DNA synthesizer to sequence through gaps and to
35   determine the sequence of the complementary strand.
Both strands of the Dex gene were completely
sequenced. The sequence data were analyzed using the

IntelliGenetics Suite of molecular programs version
S.3. The sequence is shown in Fig. 4.

Physical Characterization of the Gene Product of DS18

Purified DS18 plaques were plated (10^4 plaques/
plate) onto M-9 sucrose (pH 6) in LB top agar (pH 6)
and incubated at 30°C until polymer formation appeared
(36-48 hrs). The top agar was scraped off and soaked
in DS buffer (200mM NaOAc, pH5.2, 0.1g/l Dextran T-9,
1.0g/l Tween 80, 1.0mM $CaCl_2$, 0.04% NaN_3) to elute any
proteins. The agar was then pelleted and the supernat-
ant concentrated to approximately 50 μl. Sample buffer
(0.05M Tris-HCl pH 6.8, 1.0% SDS, 5% β-mercaptoethanol,
10% glycerol, 0.005% bromophenol blue) was added and
the samples were analyzed by SDS-PAGE. Silver staining
revealed a protein band slightly larger than the size
expected for dextransucrase (177 kd).

Comparison of DNA Sequence of DS18 Clone to Protein Sequence of Dextransucrase

Partially-purified dextransucrase protein
(Miller et al., Carbohydrate Res. (1986) 147:119-133)
was carboxymethylated and submitted to SDS-PAGE. A
dextransucrase band was observed at 177 kd and a
dextransucrase breakdown product formed a band at 158
kD. The breakdown product retains enzyme activity.

The majority of the protein preparation was
concentrated (through Speed-Vac lyophilization) 10 fold
and loaded onto four 0.5 mm 10% Laemmli SDS gels. Dex-
transucrase was separated from other contaminants by
electrophoresis of gel for 60 min at 200v. The gel was
stained for 2-4 min in 0.1% commassie blue, 47% meth-
anol, 47% water, and 6% acetic acid; then destained in
50% methanol for 4-6 min. The clearly visible dextran-
sucrase band in the upper portion of the gel was
excised and soaked in 0.5% SDS, 1mM N-ethylmorpholine,
pH 9.0 in preparation for electroelution.

Electroelution was carried out in an ISCO
elution device. Gel chips were placed in the chamber
assembled with 50kD molecular weight cutoff dialysis
tubing and electroeluted for 120 min against 1 mM N-
5   ethylmorpholine, pH 9.0 at 4°, 3 watts. Eluted sample
was recovered from the concentrating well. The well
was rinsed several times with additional buffer and
pooled with the eluted sample to make a final volume of
8 ml. Subsequent SDS-PAGE analysis established the
10   presence of a single molecular weight species at
approximately 177kD.

Aliquots (5% each) were taken and dried in
preparation for amino acid hydrolysis. One sample was
performic acid oxidized to enable identification of
15   cysteine and methionine residues. Samples were then
subjected to vapor phase HCl/phenol hydrolysis for
24 hr, 110°C in vacuo. Amino acid composition was
determined by sample application to a Beckman 6300
amino acid analyzer. (See Figure 5.)
20   Peptides were generated from one-third of the
remaining dextransucrase to facilitate determination of
protein sequence. Chemical cleavage with cyanogen
bromide was chosen to optimize the number of peptides
generated. The protein was lyophilized to dryness,
25   resuspended in 98% formic acid, then adjusted to 70%
formic acid by adding water. β-mercaptoethanol was
added to 1%. Cyanogen bromide crystals were added to a
concentration of approximately 20-30 mg/ml. The
reaction proceeded for 24 hr at room temperature, in
30   the dark at which time a sample was analyzed by SDS gel
electrophoresis. The remaining digest was size
fractionated over a HPLC TSK 2000sw sizing column in
50% acetonitrile, 0.1% TFA.

Two fractions from this sizing column were
35   each partially dried down and brought up to 100 μl in
0.1% TFA. Further fractionation of the peptides
contained in these size cuts was achieved through HPLC

chromatography on a narrow bore, reverse phase C8
column using an acetonitrile/H_2O gradient. Material
recovered from this column served as the sample for
protein sequencing.

Based on a comparison of the readable sequence
to the DNA sequence, four peptides were identified in
the sequencing sample. The four peptide sequences can
be matched to the DNA sequence. The four peptides read
as follows:

1) NFGTITAN-A-A-F

2) VDRSNDSTENEAIPNYSFV-R-D-V

3) D-Y

4) -E/GYAFLEFLQ/VAL

(dashes indicate the amino acid at the site was not
identified, slashes indicate an either/or situation).
The location of the peptide sequences in the transla-
tion of the DNA sequence is shown in Figure 5.

Example 4
Construction of Levansucrase
Expression Cassette

Plasmid pLS8 containing the B. subtilis levan-
sucrase gene was obtained from P. Gay (Gay et al., J.
Bacteriol. (1983) 153:1424-1431). The RsaI-EcoRI frag-
ment containing the 5'-end of the gene and the EcoRI-
EcoRV fragment containing the 3'-end of the gene were
isolated from polyacrylamide and agarose gels, respec-
tively, and ligated into the SmaI site of Bluescribe
M13 (minus). The resulting clones, pCGN498 and pCGN499
have the levansucrase gene inserted into the Bluescribe
polylinker in opposite directions.

Insertion of Levansucrase Gene into the mas Cassette

pCGN1047 is an expression cassette consisting
of the 5' and 3' non-coding regions of the mannopine

synthase (mas) gene (position 20804 to 20126) of Barker
et al., Plant Mol. Biol. (1983) 2:335-350) with a poly-
linker between the 5' and 3' regions. The insert from
pCGN499 was excised as a Bam-Sst fragment and ligated
5   into Bam-Sst cut pCGN1047. The resulting plasmid,
pCGN1201, contained the levansucrase coding region
between the 5' and 3' regions of the mas promoter
(Figure 12).

10   Transfer of mas-Levansucrase Construction
     into Broad Host Range Binary Plasmid
             The binary plasmid used for transfer of the
     mas-levansucrase construction to Agrobacterium was
     pCGN783 described in co-pending U.S. application serial
15   number 138,361 filed April 29, 1988 which is hereby
     incorporated by reference.

     (a)   Construction of pCGN783
             pCGN783 is a binary plasmid containing the
20   left and right T-DNA borders of A. tumefaciens octopine
     Ti-plasmid pTiA6 (Currier and Nester, J. Bacteriol.
     (1976) 126:157-165) the gentamicin resistance gene of
     pPH1J1 (Hirsch et al., Plasmid (1984) 12:139-141), the
     35S promoter of cauliflower mosaic virus (CaMV)
25   (Gardner et al., Nucleic Acid Res. (1981) 9:1871-1880);
     the kanamycin resistance gene of Tn5 (Jorgensen, Mol.
     Gen. (1979) 177:65); and the 3' region from transcript
     7 of pTiA6 (Currier and Nester, supra (1976)).

30   (b) Construction of pCGN587
             The HindIII-SmaI fragment of Tn5 containing
     the entire structural gene for APH3'II (Jorgensen et
     al., Mol. Gen. (1979) 177:65), was cloned into pUC8
     (Vieira and Messing, Gene (1982) 19:259), converting
35   the fragment into a HindIII-EcoRI fragment, since there
     is an EcoRI site immediately adjacent to the SmaI site.
     The PstI-EcoRI fragment containing the 3' portion of

the APH3'II gene was then combined with an EcoRI-BamHI-Sal1-PstI linker into the EcoRI site of pUC7 (pCGN546W). Since this construct does not confer kanamycin resis-tance, kanamycin resistance was obtained by inserting
5 the BclI-PstI fragment of the APH3'II gene into the BamHI-PstI site (pCGN546X). This procedure reassembles the APH3'II gene, so that EcoRI sites flank the gene. An ATG codon was upstream from and out of reading frame with the ATG initiation codon of APH3'II. The undesi-
10 red ATG was avoided by inserting a Sau3A-PstI fragment from the 5' end of APH3'II, which fragment lacks the superfluous ATG, into the BamHI-PstI site of pCGN546W to provide plasmid pCGN550. The EcoRI fragment of pCGN550 containing the APH3'II gene was then cloned
15 into the EcoRI site of pUC8-pUC13 (K. Buckley supra (1985)) to give pCGN551.

Each of the EcoRI fragments containing the APH3'II gene was then cloned into the unique EcoRI site of pCGN451, which contains an octopine synthase cas-
20 sette for expression to provide pCGN548 (2ATG)) and pCGN552 (1ATG). The plasmid pCGN451 having the ocs 5' and the ocs 3' in the proper orientation was digested with EcoRI and the EcoRI fragment from pCGN551 con-taining the intact kanamycin resistance gene inserted
25 with EcoRI site to provide pCGN552 having the kanamycin resistance gene in the proper orientation. This ocs/KAN gene was used to provide a selectable marker for the trans type binary vector pCGN587.

The 5' portion of the engineered octopine syn-
30 thase promoter cassette consists of pTiA6 DNA from the XhoI at bp 15208-13644 (Barker et al., supra (1983)), which also contains the T-DNA boundary sequence (border) implicated in T-DNA transfer. In the plasmid pCGN587, the ocs/KAN gene from pCGN552 provides a selectable
35 marker as well as the right border. The left boundary region was first cloned in M13mp9 as a HindIII-SmaI piece (pCGN502) (base pairs 602-2212) and recloned as a

KpnI-EcoRI fragment in pCGN565 to provide pCGN580. As
described above, pCGN565 is a cloning vector based on
pUC8-Cm, but containing pUC18 linkers. pCGN580 was
linearized with BamHI and used to replace the smaller
5   BqlI fragment of pVCK102 (Knauf and Nester, Plasmid
(1982) 8:45), creating pCGN585. By replacing the
smaller SalI fragment of pCGN585 with the XhoI fragment
from pCGN552 containing the ocs/KAN gene, pCGN587 was
obtained.

10

(c) Construction of pCGN739 (Binary Vector)
         To obtain the gentamicin resistance marker,
the resistance gene was isolated from a 3.1 kb EcoRI-
PstI fragment of pPHIJI (Hirsch et al., Plasmid (1984)
15   12:139-141) and cloned into pUC9 (Vieira et al., Gene
(1982) 19:259-268) yielding pCGN549.
         The pCGN549 HindIII-BamHI fragment containing
the gentamicin resistance gene replaced the HindIII-
BqlII fragment of pCGN587 creating pCGN594.
20   The pCGN594 HindIII-BamHI region which con-
tains an ocs-kanamycin-ocs fragment was replaced with
the HindIII-BamHI polylinker region from pUC18 (Yanisch-
Perron, Gene (1985) 33:103-119) to make pCGN739.

25   (d) Construction of 726c (1 ATG-Kanamycin-3' region)
         pCGN566 contains the EcoRI-HindIII linker of
pUC18 (Yanisch-Perron, ibid) inserted into the EcoRI-
HindIII sites of pUC13-Cm (K. Buckley, Ph.D. Thesis,
University of California, San Diego, 1985). The HindIII-
30   BqlII fragment of pNW31c-8, 29-1 (Thomashow et al., Cell
(1980) 19:729) containing ORF1 and 2 (Barker et al.,
Plant Mol. Biol. (1984) 2:335-350) was subcloned into
the HindIII-BamHI sites of pCGN566 producing pCGN703.
         The Sau3A fragment of pCGN703 containing the
35   3' region of transcript 7 from pTiA6 (corresponding to
bases 2396-2920 of pTi15955 (Barker et al., supra (1984))
was subcloned into the BamHI site of pUC18 (Yanisch-

Perron et al., supra (1985)) producing pCGN709.

The EcoRI-SmaI polylinker region of pCGN709 was replaced with the EcoRI-SmaI fragment from pCGN587 (see 6.1(a), supra) which contains the kanamycin resis-
tance gene (APH3'II) producing pCGN726.

The EcoRI-SalI fragment of pCGN726 plus the BglII-SalI sites of pUC8-pUC13-cm (chloramphenicol resistant, K. Buckley, Ph.D. Thesis, University of California, San Diego, 1985) producing pCGN738. To construct pCGN734, the HindIII-SphI site of M13mp19 (Norrander et al., Gene (1983) 26:101-106). Using an oligonucleotide corresponding to bases 3287 to 3300, DNA synthesis was primed from this template. Following S1 nuclease treatment and HindIII digestion, the resul-
ting fragment was cloned into the HindIII-SmaI site of pUC19 (Yanisch-Perron et al., supra (1985)). The re-
sulting EcoRI to HindIII fragment of pTiA6 (correspon-
ding to bases 3390-4494) was cloned into the EcoRI site of pUC8 (Vieira and Messing, supra (1982)) resulting in pCGN734. pCGN726c is derived from pCGN738 by deleting the 900 bp EcoRI-EcoRI fragment.

(e) Construction of pCGN167

pCGN167 is a construct containing a full length CaMV promoter, 1 ATG-kanamycin gene, 3' end and the bacterial Tn903-type kanamycin gene. MI is an EcoRI fragment from pCGN550 (see construction of pCGN587) and was cloned into the EcoRI cloning site in the 1 ATG-
kanamycin gene proximal to the polylinker region of M13mp9. See copending Application Serial No. 920,574, filed October 17, 1986, which disclosure is incorpora-
ted herein by reference.

To construct pCGN167, the AluI fragment of CaMV (bp 7144-7735) (Gardner et al., Nucl. Acids Res. (1981) 9:2871-2888) was obtained by digestion with AluI and cloned into the HincII site of M13mp7 (Vieira, Gene (1982) 19:259) to create C614. An EcoRI digest of C614

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produced the EcoRI fragment from C614 containing the
35S promoter which was cloned into the EcoRI site of
pUC8 (Vieira et al., Gene (1982) 19:259) to produce
pCGN146.   To trim the promoter region, the BglII site
5   (bp 7670) was treated with BglII and Bal31 and subse-
quently a BglII linker was attached to the Bal31 trea-
ted DNA to produce pCGN147.

pCGN148a containing the promoter region,
selectable marker (KAN with 2 ATGs) and 3' region was
10   prepared by digesting pCGN528 (see below) with BglII
and inserting the BamHI-BglII promoter fragment from
pCGN147.   This fragment was cloned into the BglII site
of pCGN528 so that the BglII site was proximal to the
kanamycin gene of pCGN528.

15   The shuttle vector used for this construct,
pCGN528, was made as follows.   pCGN525 was made by
digesting a plasmid containing Tn5 which harbors a
kanamycin gene (Jorgenson et al., Mol. Gen. (1979)
177:65) with HindIII-BamHI and inserting the HindIII-
20   BamHI fragment containing the kanamycin gene into the
HindIII-BamHI sites in the tetracycline gene of
pACYC184 (Chang and Cohen, J. Bacteriol. (1978) 134:
1141-1156).   pCGN526 was made by inserting the BamHI
fragment 19 of pTiA6 (Thomashow et al., Cell (1980)
25   19:729-739) into the BamHI site of pCGN525.   pCGN528
was obtained by deleting the small XhoI fragment from
pCGN526 by digesting with XhoI and religating.

pCGN149a was made by cloning the BamHI kana-
mycin gene fragment from pMB9KanXXI into the BamHI site
30   of pCGN148a.   pMB9KanXXI is a pUC4K variant (Vieira and
Messing, Gene (1982) 19:259-268) which has the XhoI site
missing but contains a functional kanamycin gene from
Tn903 to allow for efficient selection in Agrobacterium.

pCGN149a was digested with BglII and SphI.
35   This small BglII-SphI fragment of pCGN149a was replaced
with the BamHI-SphI fragment from MI isolated by
digestion with BamHI and SphI.   This produces pCGN167.

(f) <u>Construction of pCGN766c</u> (35S promoter-3' region)

The <u>Hind</u>III-<u>Bam</u>HI fragment of pCGN167 containing the CaMV-35S promoter, 1 ATG-kanamycin gene and the <u>Bam</u>HI fragment 19 of pTiA6 was cloned into the <u>Bam</u>HI-<u>Hind</u>III sites of pUC19 (Norrander <u>et al.</u>, <u>supra</u> (1985); Yanisch-Perron <u>et al.</u>, <u>supra</u> (1985)) creating pCGN976.

The 35S promoter and 3' region from transcript 7 was developed by inserting a 0.7 kb <u>Hind</u>III-<u>Eco</u>RI fragment of pCGN976 (35S promoter) and the 0.5 kb <u>Eco</u>RI-<u>Sal</u>I fragment of pCGN709 (transcript 7:3' for construction see <u>supra</u>) into the <u>Hind</u>III-<u>Sal</u>I sites of pCGN566 creating pCGN766c.

(g) <u>Final Construction of pCGN783</u>

The 0.7 kb <u>Hind</u>III-<u>Eco</u>RI fragment of pCGN766c (CaMV-35S promoter) was ligated to the 1.5 kb <u>Eco</u>RI-<u>Sal</u>I fragment of pCGN726c (1-ATG-KAN-3' region) into the <u>Hind</u>III-<u>Sal</u>I sites of pUC119 (J. Vieira, Rutgers University, New Jersey) to produce pCGN778. The 2.2 kb region of pCGN778, <u>Hind</u>III-<u>Sal</u>I fragment containing the CaMV 35S promoter (1-ATG-KAN-3' region) replaced the <u>Hind</u>III-<u>Sal</u>I polylinker region of pCGN739 to produce pCGN783.

The <u>mas</u>-levansucrase cassette pCGN1201 was inserted into the unique <u>Sal</u>I site of pCGN783 to create plasmid pCGN1205. This plasmid in <u>E. coli</u> C2110 is conjugated into <u>Agrobacterium</u> strain PC2760 (Ooms <u>et al.</u>, <u>Plasmid</u> (1982) <u>7</u>:15-29; Hoekema <u>et al.</u>, <u>Nature</u> (1983) <u>303</u>:179-181; European Patent Application 84-200239; 2424283) which contains a disarmed Ti-plasmid, using gentamicin selection.

Example 5
Construction of Dextransucrase
Expression Cassettes

Expression of Cassettes Generally

The dextransucrase gene from pCGN1222 was
cloned through a series of steps into pCGN2306 (Figure
8). The dextransucrase gene was then removed from
pCGN2306 as an EcoRV fragment and cloned into the
respective promoter cassettes (pCGN2305, having the 35S
CaMV promoter and pCGN1241, having the 2All
promoter). The resulting constructs were then cloned
into a binary cassette (pCGN1547) in both orientations,
yielding a total of 4 constructs, pCGN2318 and 2319 and
pCGN2310 and 2311 (Figure 9).

2All Promoter Cassette

The design of the 2All cassette (pCGN1241) is
shown in Figure 7. The cassette contains 3.8 kb of DNA
5' of the transcriptional start site and the entire 3'
region (from the TGA stop codon to a site 2.0 kb 3' of
the poly A addition site) of the 2All gene. A map of
the 2All gene is shown in Figure 6, showing the
restriction sites and indicating (below the
representation of the gene) the regions of the 2All
gene used to construct the 2All cassette. The 2All
cassette was constructed as follows.

The 5' end of the 2All cassette was
constructed starting with an EcoRI subclone genomic
clone as described in application PCT/US88/01811 cloned
into the EcoRI site of Bluescript (+) (Stratagene)
resulting in pCGN1288. This clone contains sequences
from the EcoRI site at position 1651 in the intron of
the 2All gene to the EcoRI site located 2.5 Kb upstream
of the XhoI site at position 1 of the sequenced region
(see Figure 6). The XhoI fragment from position 1 of
the sequenced region to the XhoI site in the Bluescript

polylinker was deleted creating plasmid pCGN2004 which
contains the 2A11 region from position 1 to position
1651. The coding region of 2A11 was deleted by
treating this plasmid with ExonucleaseIII/S1 (using the
5   commercially available Erase-a-Base Kit (Promega
Biotec)) and sequencing deletion plasmids until one was
found which had the coding region deleted to position
1366. The resulting plasmid, pCGN1251, had the genomic
region from the XhoI site (position 1) to position
10   1366. The EcoRI fragment of pCGN1288 was then
transferred to a chloramphenicol resistant plasmid
vector, pCGN2015, to make pCGN1231. pCGN2015 is a Cm
resistant derivative of the Bluescript plasmid. A
BstEII/BamHI fragment of pCGN1251 was then transferred
15   into BstEII/BamHI digested pCGN1231 to make pCGN1235
which contains the region from the EcoRI site (2.5 kb
upstream of the sequenced region) to position 1366 of
the sequenced region flanked by the Bluescript
polylinker in a Cm resistant vector.
20   The 3' end of the 2A11 cassette was
constructed from pCGN1273 (described in application
PCT/US88/01811) by digesting the plasmid with PvuI and
EcoRI, isolating the 2249 bp insert (from position 2402
to 4653), ligating with a double-stranded
25   oligonucleotide containing the sequence shown in Figure
6 from the BamHI sticky end to a PvuI sticky end into a
Bluescript vector which had been digested with BamHI
and EcoRI. The resulting plasmid, pCGN1238 contains
the 3' end of the 2A11 gene from the stop codon at
30   position 2381 to the EcoRI site at position 4653.
         A cassette containing the 5' and 3' regions of
the 2A11 gene was constructed by ligating the BamHI to
EcoRV insert of pCGN1238 into pCGN1235 which had been
digested with BamHI and XbaI (the XbaI site having been
35   filled in with Klenow polymerase to make a blunt-ended
fragment). The resulting plasmid, pCGN1240, has the 5'
end of the 2A11 gene from the EcoRI site 2.5 kb

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upstream of the XhoI site (position 1) to position 1366
(which is located between the transcriptional
initiation site of the 2All gene and the ATG), followed
by a polylinker region (sequence given in Figure 6)
5   with sites for SmaI, BamHI, PstI and SalI which can be
conveniently used to insert genes followed by the 3'
region from position 2381 to 4653. The plasmid
backbone of pCGN1240 is the Bluescript Cm plasmid
described above. A more convenient version has the
10   EcoRI of 1240 excised and inserted into a Bluescript
vector with an altered polylinker from the SacI site to
the KpnI site with a synthetic polylinker with the
following sequence:

             AGCTCGGTACCGAATTCGAGCTCGGTAC to create a

15   _polylinker with the following sites: SacI-KpnI-SacI-
KpnI. The EcoRI insert of pCGN1240 was inserted into
pCGN1240 to make pCGN1241 (Figure 7).

35S Promoter Cassette

20   The 35S CaMV cassette (pCGN2305) contains DNA
5' of the transcriptional start site of the 35S CaMV
gene of the 3' region of the Tr7 gene. It was
constructed as follows. pCGN1410 (Sheehy et al.,
(1988) Proc. Natl. Acad. Sci. (USA), 85:8805-8809) is
25   digested with EcoRI, and the ends filled in with DNA
polymerase I. A phoshorylated XbaI linker from
Pharmacia, 5'-pd[TCTAGA]-3' is ligated in. The
resulting plasmid was called pCGN2304. pCGN2304 was
then digested with HindIII, the ends filled in with DNA
30   polymerase I, and a phosphorylated XbaI linker ligated
in. The resulting plasmid was called pCGN2305.

Preparation of Necessary Constructs

          pCGN2302. The 3' end of the gene was
subcloned from pCGN1222 (see Example 3) as a 3.1 kb
35   SalI/EcoRV fragment into pCGN2015 to give pCGN2302.
The plasmid pCGN2015 was constructed by digesting

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pCGN565 with HhaI blunting with mung bean nuclease and ligating with EcoRV digested Bluescript KSM13 (Stratagene, San Diego, CA) to create pCGN2008. pCGN565 is a cloning vector based on pUC8-pUC13-cm (K.
5 Buckley (1985), supra) but containing pUC18 linkers (Yanisch-Perron et al., Gene (1985) 53:103-119).

        pCGN2008 was digested with EcoRI and HindIII, blunted with Klenow. The 1156 bp chloramphenicol fragment was isolated. Bluescript KSM13+ (Stratagene)
10 was digested with DraI and the 2273 bp fragment is isolated and ligated with the pCGN2008 chloramphenicol fragment creating pCGN2015.

        pCGN2306. The remaining 1.6 kb 5' end of the dextransucrase gene was generated using the polymerase
15 chain reaction method, Taq polymerase (Gene Amp kit) and the DNA thermal cycler (Perkin-Elmer Cetus) using pCGN2023 as a template. pCGN was made by a BamHI deletion of pCGN1222. Two oligonucleotides, Dex02 (42-mer with the added restriction sites, XhoI and EcoRV
20 upstream of the ATG codon, plus sequence complementary to the ATG region) and Dex03 (a 26-mer complimentary to the SalI region) were used as primers.Dex02: 5'-GGTTATCTCGAGGATATCATGCCATTTACAGAAAAGTAATG-3'
Dex03: 5'-CAAATCAGCATCCACATTGTCGACTG-3'
25   The resulting 1.6 kb fragment contains the 5' end of the gene, from the ATG start codon through the SalI site with an additional XhoI site and an EcoRV site upstream of the ATG. The 1.6 kb fragment was then cloned in pCGN2303 as an XhoI/SalI fragment, in front
30 of the 3' end of the gene giving plasmid pCGN2306.

        pCGN1547. pCGN1547 (described in co-pending U.S. patent application Serial No. 329,018, (which disclosure is hereby incorporated by reference) is a binary plant transformation vector containing the left
35 and right T-DNA borders of Agrobacterium tumefaciens octopine Ti-plasmid pTiA6 (Currier and Nester, J. Bact. (1976) 126:157-165), the gentamycin resistance gene of

pPH1JI (Hirsch and Beringer, Plasmid (1984) 12:139-
141), an Agrobacterium rhizogenes Ri plasmid origin of
replication from pLJbBll (Jouanin et al., Mol. Gen.
Genet. (1985) 201:370-141), the mas promoter region and
5   mas 3' region of pTiA6 with the kanamycin resistance
gene of Tn5 (Jorgensen et al., Mol. Gen. Genet. (1979)
177:65), a ColEl origin of replication from pBR322
(Bolivar et al., Gene 2:95-113), and a lacZ' screenable
marker gene from pUC18 (Yannisch-Perron et al., Gene
10   33:103-119).

Final Construction Steps

The entire dextransucrase gene was removed
from pCGN2306 as an EcoRV fragment and cloned as a
blunt ligation into the SmaI sites of the promoter
15   cassettes pCGN2305, giving plasmid pCGN2309, and
pCGN1241, giving pCGN2307. Each cassette was cloned
into the binary vector pCGN1547 as an XbaI fragment for
the 35S cassette to create pCGN2318 and pCGN2319, and
as a KpnI fragment fro the 2All cassette to create8
20   pCGN2310 and 2311. This allows for constructs in both
orientations for each cassette. The completed binaries
were then submitted for cocultivation. Agrobacterium
tumefaciens strain 2760 (also known as LBA 4404,
Hoekema et al., Nature (1983) 303:179-180) were
25   transformed with the receptive binary using the method
of Holsters et al. Mol. Gen. Genet. (1978) 163:181-
187. The transformed binary was then used in the
cocultivation of plants.

Example 6
30   Preparation of Transgenic Plants

Feeder plates were prepared by pipetting 0.5
ml of an eight day old suspension of Nicotiana tabacum
cv xanthi cell suspension culture (~10^6 cells/ml) onto
35   0.8% agar medium, containing MS salts, myo-inositol
(100 mg/l), thiamine-HCl (1.3 mg/l), sucrose (30 g/l),
potassium acid phosphate (200 mg/l) 2,4-D (0.2 mg/l),

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and kinetin (0.1 mg/l) (pH 5.5). The feeder cells were
prepared at least 24 hours prior to use. A #1 Whatman
sterile filter paper (Whatman Ltd, Maidstone, England)
was placed on top of the tobacco feeder cells after the
5   cells had been growing for at least 24 hours.

Agrobacteria containing the plasmid of
interest were grown on AB medium (AB salts K_2HPO_4 3
gm/l, NaH_2PO4 H_2O 1.15 g/l, NH_4Cl 1 g/l, glucose 5 g/l,
$FeSO_4$ 0.25 mg/l, $MgSO_4$ 0.246 mg/l, 0.14 mg/l, 15 g/l
10   agar 100 u/l gentamycin sulfate and 100 u/l
streptomycin sulfate) for 4-5 days. Single colonies
were then inoculated into 5 mls of MG/L broth and
preincubated overnight in a shaker (180 rpm) at 30°C.

Sterile tomato cotyledon tissue was obtained
15   from 7-8 day old seedlings which had been grown at
24°C, with a 16hr/ 8hr day/night cycle in 100 x 25 mm
petri dishes containing MSSV medium: Murashige-Skoog
(MS) salts (#1117 Gibco Laboratories, New York),
sucrose 30 g/l, Nitsch vitamins (Thomas, B.R., and
20   Pratt, D. Appl. Genet. (1981) 59:215-219), 0.8% agar
(pH 6.0). Any tomato species may be used, however, the
inbred breeding line UC82B (Department of Vegetable
Crops, University of California, Davis) is preferred.
The tips and bases of the cotelydons were removed and
25   the center section placed onto a feeder plate for a 24-
hour preincubation period in a low light, 40-50
microEinsteins, but no greater than 80 microEinsteins,
at 24°C.

Following the preincubation period, the
30   cotyledon explants were then dipped into the
agrobacteria suspension (5 x 10^8 bacteria/ml) for
approximately 5 minutes, blotted on sterile paper
towels and returned to the original tobacco feeder
plates. The explants were cocultivated with the
35   agrobacteria for 48 hours on the tobacco feeder plates
in low light (see above) at 24°C, then transferred to
regeneration medium containing 500 mg/l of carbenicil-

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lin disodium salts and at least 100 mg/l of kanamycin
sulfate. The regeneration medium is MS sa4s medium
with zeatin (2 mg/l), myo-inositol (100 mg/l), sucrose
(20 g/l), Nitsche vitamins and 0.8% agar (pH 6.0).
5   After 10 days and subsequently every three weeks, the
explants were transferred to fresh regeneration medium
containing 500 mg/l of carbenicillin disodium salts and
at least 100 mg/l of kanamycin sulfate. Shoots were
harvested from 8 weeks onwards and placed on MSSV
10   medium containing carbenicillin (50 mg/l), kanamycin
(50 mg/l) and indole-3-butyric acid (1 mg/l). Roots
developed in 7-14 days. Plants were then transplanted
into soil.

15

Example 7
Targeting to Apoplast

          The hydropath plot of the deduced amino acid
20   sequence of the Leuconostoc dextransucrase shows a
highly hydrophilic protein with a hydrophobic
N-terminal region. As seen with many other gram-
positive secreted proteins, the N-terminal sequence
displays characteristics expected of a signal
25   peptide. However, because these sequences are quite
different from those seen for eukaryotic secretory
proteins, it is possible that a heterologous leader
sequence will be desired for efficient translocation of
the sucrase enzyme to a desired cell organelle.
30   Various constructs therefore have been designed to
optimize targeting of dextransucrase to the fruit
apoplast.

PG/Dextransucrase Constructs
35   Constructs using the full-length dextransu-
crase gene as well as various 5' deletions of the gene
were fused to either the full length polygalacturonase

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(PG) leader (3141bp) or the 5' region of the
polygalacturonase leader (146 bp). A new dextransu-
crase construct was made using the polymerase chain
reaction (PCR) method. A 1.6 kb XhoI-SalI fragment of

5   pCGN1222 was synthesized and inserted into XhoI
digested pCGN2303 to make plasmid pCGN2327. This new
construct, pCGN2327, Figure 10, is identical to
pCGN2306 but lacks the EcoRV site at the 5' end.

       Constructs containing the PG leaders fused to

10   dextransucrase were prepared as follows: The full
length PG leader fragment was cloned as a 314 bp XhoI-
SalI PCR fragment into the XhoI site of pCGN2327 to
give pCGN2330. The 5' PG leader fragment was cloned as
a 146 bp XhoI-SalI PCR fragment into the XhoI site of

15   pCGN2327 to give pCGN2331 (Figure 11). Other
constructs having either the full-length or a shorter
5' fragment of the PG-leader are fused to the relevant
sucrase genes in a like manner. Where it is desirable,
for example, to delete 5' bacterial transit peptide

20   regions, portions of the sucrase gene can be deleted to
increase activity of the encoded protein. Once the
construct is prepared having the desired truncations
and/or deletions, the PG leader/dextransucrase regions
can then be cloned into the 2A11 and 35S promoter

25   cassettes (pCGN1241 and pCGN2305 respectively) and then
into the binary plasmid 7, or other such constructs.

       The resulting binary plasmid having the
desired constructs therein are used to transform A.
tumefaciens and through Agrobacterium-mediated trans-

30   formation, to obtain transgenic plants.

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Example 8
Analysis of Constructs
Comprising Transit Peptides

5   Two PG leader/GUS constructs were made to
study the targetting of dextransucrase. The two
constructs, pCGN2313 (full length PG leader) and
pCGN2325 (5' PG leader) (Fig. 13), were electroporated
into tobacco protoplasts and all fractions (media, cell
10   wall debris, and protoplast) were assayed for GUS
activity. (Jefferson et al. Plant Molec. Biol. Rep.
(1987) 5:387-405.) In initial studies, no GUS activity
was detected in any fraction. The GUS gene contains
two glycosylation sites. It has been proposed that
15   glycosylation of GUS at one or more of these sites
during processing may inactivate the enzyme.
Therefore, the electroporation studies were repeated
using the antibiotic tunicamycin, which interferes with
glycoprotein biosynthesis and prevents glycosylation of
20   GUS. Data from these studies (Table 9) show that when
tunicamycin is added, GUS activity is retained and
glycosylation appears blocked. Since glycosylation of
proteins occurs between the endoplasmic reticulum and
the Golgi, the PG leader, full length as well as 5'
25   only, appears to be successfully taking the GUS protein
into the secretory pathway. This is also supported by
the fact that the GUS activity is reaching the cell
wall fraction of the electroporated protoplasts.
Western analysis of the protplast fractions will be
30   used to verify these results.

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Table 9[1]

GUS Activity In
Electroporated Protoplasts

Construct	Without Tunicamycin Cell Wall		With Tunicamycin Cell Wall	
	Protoplast	Debris	Protoplast	Debris
pBI221[2]	2840	471	23450	431
	20250	214	17500	309
pCGN2313	990	104	25700	9590
	940	70	27400	5475
pCGN2325	2660	192	59100	9990
	1180	101	58300	14070

[1]GUS activity is assayed as described by Jefferson et al. (supra). Activity is reported as fluorescence/180 min. reaction/electroporation (fluorescence units at 365 nm excitation/455 nm emission).

[2]pBI221 is a 35S/GUS construct used as a positive control.

Analysis of PG Leader-Dextransucrase

Transformed Tissue

Localization of dextransucrase in the apoplast of the tomato fruit can be determined by various methods. One such method is tissue printing of the fruit onto nitrocellulose paper (Cassab and Varner, (1987) J. Cell. Biol., 105:2581-2588). In this procedure a transformed tomato fruit is sliced, washed briefly in distilled water, quickly dried and blotted onto nitrocellulose paper that has previously been soaked in 0.2M $CaCl_2$ and dried. The tissue print is then immediately dried and treated for the detection of dextransucrase using alkaline phosphatase-conjugated second antibody as follows. The nitrocellulose blot is incubated in a blocking buffer consisting of 50 mM Tris-HCl, pH7.5, 200mM NaCl, 0.05% Tween-20 (TTBS) and

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1% bovine serum albumin, then incubated in rabbit
antidextransucrase antibody in TTBS. Biotinylated goat
anti-rabbit IgG is then bound followed by incubation in
streptavidin-alkaline phosphatase conjugate. BCIP (5-
5 bromo-4-chloroindoxyl phosphate) and NBT (nitroblue
tetrazolium) are used as substrate to visualize the
precipitated indoxyl group. Individual cells on the
tissue print are then examined under a dissecting
microscope for localization of dextransucrase.

10   Another method used to locate dextransucrase
in transformed tomato fruit is immunofluoresence
(Vreeland et al., (1984), Planta., 162:506-517) or
immunogold (Mauch and Staehelin, (1989), Plant Cell.,
1:447-457, Dorel et al., (1989), J. Cell Biol.,
15 108:327-337, Cassab and Varner, (1987), J. Cell Biol.,
105:2581-2588) labelling of antigen in thin tissue
sections by dextransucrase polyclonal antibody.
Transformed tomato fruit are dissected under fixative
conditions appropriate for either light or electron
20 microscopic immunocytochemistry (Greenwood and
Chrispeels, (1985), Plant Physiol., 79:65-71). Thin
sections of the fruit (0.05-1um) then are cut from the
fixed tissue, mounted on slides and processed for
immunocytochemistry by either immunofluoresence or
25 immunogold labelling techniques. The labelled thin
sections are then analyzed under appropriate
microscopic conditions for the localization of
dextransucrase.

30   Example 9
           Analysis of Transgenic Plants

NPT II Enzyme Assay
         Leaves from plants transformed with a con-
35 struct of interest which regenerated from kanamycin-
resistant explant tissue were assayed for expression of
the NPT II gene. Tissue was harvested, frozen in

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liquid nitrogen, ground frozen in Eppendorf tubes
containing equivalent amounts (w/v) of extraction
buffer (2.5 mM Tris-HCl, pH 6.8, 0.143 mM ß-mercapto-
ethanol, 0.27 mM leupeptin) then centrifuged for 15 min.
5   The supernatant from leaf homogenates was added to half
volume aliquots of reaction buffers A and B. Reaction
buffer A contained 67 mM Tris-maleate, 42 mM $MgCl_2$, 400
mM NH_4Cl, 1.7 mM dithiothreitol, 0.4 mg/ml kanamycin
sulfate. Reaction buffer B was identical to buffer A,
10 except that kanamycin was omitted. $[\gamma-^{32}P]$ ATP solu-
tion (0.75 mM ATP dissolved in reaction buffer B and
1.0 uCi $[\gamma-^{32}P]$ ATP/sample) was added to each reaction
mixture. The samples were incubated at 32°C for 30
min. Using a slot blotting apparatus, reaction samples
15 were blotted onto three layers of Whatman P81 ion ex-
change papers placed on top of one Whatman 3mm paper.
All three P81 papers were washed twice with water at
25°C for 4 min each. The blots were incubated for 45
min at 65°C in proteinase K solution (1.0 mg/ml pro-
20 teinase K in 1% SDS), then washed once at 80°C for 4
min followed by a wash at room temperature. After dry-
ing, blots were exposed to X-ray film with an intensi-
fying screen.

25 Northern Analysis

        Out of 19 transformed plants containing the
levansucrase construct, and 2 transformed plants
containing the 2All/dextransucrase construct, and 42
transformed plants containing the 35S/dextransucrase
30 construct, 14, 41 and 35 plants, respectively, show
expression the kan gene.

Isolation of Poly(A)$^+$ RNA

        Leaves and fruit (green as well as ripe) from
35 transformed tomato plants were harvested and frozen in
liquid nitrogen. Frozen leaves were ground in a mortar
and pestle in liquid nitrogen and total RNA was extrac-

51

ted from the resulting powder by homogenization in
Tris-HCl, SDS buffer and lithium chloride precipitation
as described by Crouch et al., J. Mol. Appl. Gen. (1983)
2:273-283. Frozen ripe fruit was ground to a powder in
5   a mortar and pestle in liquid nitrogen and homogenized
with a Brinkmann polytron continuously for 2 min on
ice. Total RNA is isolated as described by Colbert et
al., Proc. Natl. Acad. Sci. (1983) 80:2248-2252 using
the extraction buffer described by Facciotti et al.,
10   Bio/Tech (1985) 3:241-246 and pelleted through a high-
density CsCl solution on a Beckman type 60Ti rotor as
described by Turpen and Griffith, BioTechniques (1986)
4:11-15. Poly(A)$^{+}$ RNA is isolated as described by
Maniatis et al. (1982). Contaminating sucrases are
15   removed from the samples by washing with Sigmacell ^{R} type
50 in 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM EDTA, 0.1%
SDS.

RNA Probe Preparation
20   A ^{32}P-labeled RNA probe complementary to the gene
of interest is synthesized using a Riboprobe kit (Promega
Biotech), according to the manufacturers instructions, and
labeling with a^{32}P-rUTP, according to the manufacturer's
instructions. The probe to the levansucrase gene was
25   synthesized from the T7 promoter of the plasmid pCGN499
(see supra).

Analysis
            mRNA isolated from leaves of transformed plants
30   is denatured and electrophoresed as described by Shewmaker
et al., Virology (1985) 140:281-288. The gels were
blotted overnight against 20x SSC (3 M sodium chloride,
0.3 M sodium citrate, pH 7.0) the nitrocellulose filters
baked at 80°C for 2 hrs and then hybridized at 50°C in the
35   hybridization buffer suggested by the Riboprobe manufac-
turer containing 0.5 mg/ml denatured yeast tRNA, 0.5 mg/ml
denatured salmon sperm DNA, and 0.25 mg/ml polyanethole-

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sulfonic acid. Blots are then washed at 55°C for 30 min
and twice at 65°C for 30 min each in buffer as described
by the Riboprobe manufacturer. Contaminating ribosomal
RNA is removed from the blots by washing them with 1 ug/ml
5 ribonuclease in 2x SSC at 25°C.

As shown in Figure 12, two of the transformed
plants analyzed hybridized to the levansucrase probe,
although only one of the constructs was producing a full
length transcript. Analysis of mRNA from ripe fruit from
10 the transformants gave the same results.

Analysis of Tomato Fruit for Sucrase Activity

Protein is isolated from green fruit from a
transformed plant, which appears from Northern analysis to
15 be producing a full length transcript of the gene of
interest, and from green fruit of nontransformed Calgrande
plants as a negative control. The method used is as
follows. Fruit is frozen then allowed to thaw in 1.5 M
NaCl, 0.1 M sodium citrate, pH 6 buffer, ground in a
20 mortar and pestle, then homogenized using a Polytron for
3 min at 4°C. Large debris is removed by centrifugation
twice at 5500 x g. The supernatant is then brought to 95%
$(NH_4)_2SO_4$. After stirring for 1 hr at 4°C the samples are
centrifuged and the protein precipitate is resuspended in
25 10 ml of 0.05 M KPO_4 buffer, pH 5. The samples are then
dialyzed overnight against 0.05 M KPO_4, pH 6, centrifuged
to remove any precipitants and the supernatant analyzed
for levansucrase activity.

Levansucrase was isolated from Bacillus subtilis
30 strain QB118 as described by Dedonder, Methods in Enzymol.
(1966) 8:500-505. Briefly, the supernatant from a one-
liter 14-hr culture grown in EcLB (5 g/l yeast extract,
10 g/l tryptone, 5 g/l NaCl) containing 10 g/l glucose at
30°C was isolated and pH adjusted to 4.2 by addition of
35 acetic acid. Cold ethanol (2-4°C) was gradually added to
48% and stirred continuously at 4°C for 20 min then cen-
trifuged to pellet the proteins. The pellet was dissolved

53

in 30 ml of 0.05 M KPO_4 and dialyzed overnight against 50%
$(NH_4)_2SO_4$, pH 5, at 4°C overnight. The sample was then
centrifuged and the supernatant dialyzed against 65%
$(NH_4)_2SO_4$, pH 5, at 4°C overnight. Again the sample was
5   centrifuged and the supernatant was dialyzed against 95%
$(NH_4)_2SO_4$, pH 5, at 4°C overnight. The sample was then
centrifuged to pellet the proteins and the pellet was
resuspended in 5 ml of 0.05 M KPO_4, pH 5, and dialyzed
against 0.05 M KPO_4, pH 5, at 4°C overnight.

10   Dextransucrase process a radioactive polymer only
from sucrose labelled in the glucose molecule of sucrase,
while levansucrase produces a radioactive polymer only
from sucrose labeled in the fructose molecule of
sucrose. The protocol for listing labelled [glucose-^{14}C]
15 as required in analysis of dextransucrase is discussed
above. 50 µl of the supernatant obtained as above was
added to reaction tubes containing 0.1µCi of dried sucrose
[fructose-^{14}C] and 50µl of reaction buffer (0.05M KPO_4,
pH6, 0.01M sucrose, 20mg/ml levan (10,000 MW). The
20 reaction tubes were incubated at 30°C and sampled at
various times for the incorporation of ^{14}C into polymer by
spotting aliquots of each reaction onto Whatman No. 1
filter paper. The filters were then washed in methanol as
described supra for analysis of dextransucrase.

25   The above results demonstrate that plant species
can be transformed efficiently with expression cassettes
which provide for expression of a heterologous gene for a
carbohydrate polymer synthesizing enzyme such as a
bacterial dextran or levansucrase. As evidenced by the
30 above disclosure, plant species are provided which express
a bacterial levansucrase or dextransucrase gene and as a
result may have modified solids content without adverse
effects on the normal production capabilities of the
plant.

35   All publications and patent applications
mentioned in this specification are indicative of the
level of skill of those skilled in the art to which this

54

invention pertains. All publications and patent
applications are herein incorporated by reference to the
same extent as if each individual publication or patent
application was specifically and individually indicated to
5   be incorporated by reference.

      The invention now being fully described, it will
be apparent to one of ordinary skill in the art that many
changes and modifications can be made thereto without
departing from the spirit or scope of the appended claims.
10

WHAT IS CLAIMED IS:

1.   A method for modifying the soluble solids composition of a plant cell, said method comprising:

growing plant cells comprising at least one copy of a foreign gene encoding an enzyme capable of polymerizing a carbohydrate comprising at least one hexose moiety to form a polymer under conditions whereby said gene is expressed and the soluble solids composition of said plant cells is modified.

2.   The method according to Claim 1, wherein said carbohydrate is sucrose.

3.   The method according to Claim 2, wherein said enzyme is dextransucrase or levansucrase.

4.   A method for modifying the soluble solids composition of fruit, said method comprising:

growing a plant comprising cells containing at least one copy of an expression cassette comprising, in the 5'-3' direction of transcription, a transcriptional and translational initiation region active in a fruit cell, a DNA sequence encoding an enzyme capable of polymerizing a carbohydrate comprising at least one hexose moiety to form a polymer, wherein expression of said DNA sequence is regulated by said initiation region, and translational and transcriptional termination regions functional in a plant cell, under conditions whereby said DNA sequence is expressed and the soluble solids composition of said fruit is modified.

5.   A plant cell capable of producing sucrase at an enhanced level, said cell comprising:

at least one copy of a sucrase gene, wherein said cell was obtained by inserting into said cell, or a parent of said cell, an expression cassette, prepared in vitro,

comprising a sucrase gene under the transcriptional and
translational control of transcriptional and translational
initiation and termination regulatory regions functional
in said cell.

5

        6.   The plant cell according to Claim 5, wherein
said sucrase gene is a levansucrase gene or a
dextransucrase gene.

10   7.   The plant cell according to Claim 5, wherein
said plant cell is a fruit cell or a seed cell.

        8.   The plant cell according to Claim 6, wherein
said plant cell is a tomato plant cell.

15

        9.   A plant producing increased levels of
sucrase, said plant consisting essentially of:
        cells obtained by inserting into said cells, or
parents of said cells, an expression cassette, prepared in
20   vitro, comprising a sucrase gene under the transcriptional
and translational control of transcriptional and
translational initiation and termination regulatory
regions functional in said cells, whereby said cells
produce increased levels of sucrase.

25

        10.   The plant according to Claim 9, wherein said
sucrase gene is a levansucrase gene or a dextransucrase
gene.

30   11.   A tomato plant comprising cells producing
levansucrase or dextransucrase.

        12.   The plant according to Claim 11, wherein
said cells are fruit cells.

35

        13.   An expression cassette comprising a
transcriptional and translational region functional in a

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plant cell, a DNA sequence encoding levansucrase, and a
transcriptional termination regulatory functional in a
plant cell.

5   14. A plant cell comprising an expression
cassette of Claim 13.

          15. A DNA fragment at least substantially
homologous with a DNA sequence encoding dextransucrase
10   obtainable from L. mesenteroides.

          16. A DNA fragment comprising L. mesenteroides
dextransucrase structural gene.

15   17. A DNA fragment comprising the DNA sequence
shown in Figure 4.

          18. The DNA fragment according to any one of
Claims 15, 16, or 17, wherein said fragment is joined to a
20   heterologous DNA sequence.

          19. A DNA construct comprising a DNA sequence
encoding dextransucrase obtainable from L. mesenteroides
joined to a heterologous DNA sequence.

25

          20. The DNA construct according to Claim 19,
wherein said heterologous DNA sequence is a
transcriptional and translational initiation region
functional in a plant cell.

30

          21. The DNA construct according to Claim 20,
wherein said transcriptional and translational initiation
region is active at or immediately after anthesis and
during fruit ripening.

35

          22. The DNA construct according to Claim 21,
wherein said initiation region is the 2A11 initiation
region.

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23.   An expression cassette comprising:
in the 5'-3' direction of transcription, a transcriptional
and translational region derived from 2All; a first DNA
5   sequence encoding dextransucrase or levansucrase, and a
transcriptional and translational termination regulatory
region functional in a plant cell.

24.   An expression cassette according to Claim
10   23, further comprising:
a second DNA sequence encoding a transit peptide
joined in reading frame at the 5' terminus of said first
DNA sequence, wherein said second DNA sequence is derived
from a gene encoding an apoplast polypeptide.
15

25.   An expression cassette according to Claim
24, wherein said apoplast polypeptide is tomato poly-
galacturonidase.

20   26.   A cell comprising a DNA construct according
to Claim 25.

27.   The cell according to Claim 26, wherein said
cell is a plant cell.
25

28.   The cell according to Claim 27, wherein said
plant cell is in vivo.

29.   The cell according to Claim 28, wherein said
30   plant is tomato.

30.   The cell according to Claim 29 comprising:
a DNA construct according to any one of Claims
20, 21, 22, 23, 24, and 25.
35

31. A polypeptide comprising:

a sucrase joined to a plant apoplast transit peptide sequence.

5   32. The polypeptide according to Claim 31, wherein said sucrase is dextransucrase or levansucrase.

33. The polypeptide according to Claim 32, wherein said dextransucrase is L. mesenteroides
10   dextransucrase.

34. The polypeptide according to Claim 31, wherein said plant apoplast transit peptide sequence is derived from tomato polygalacturonase.
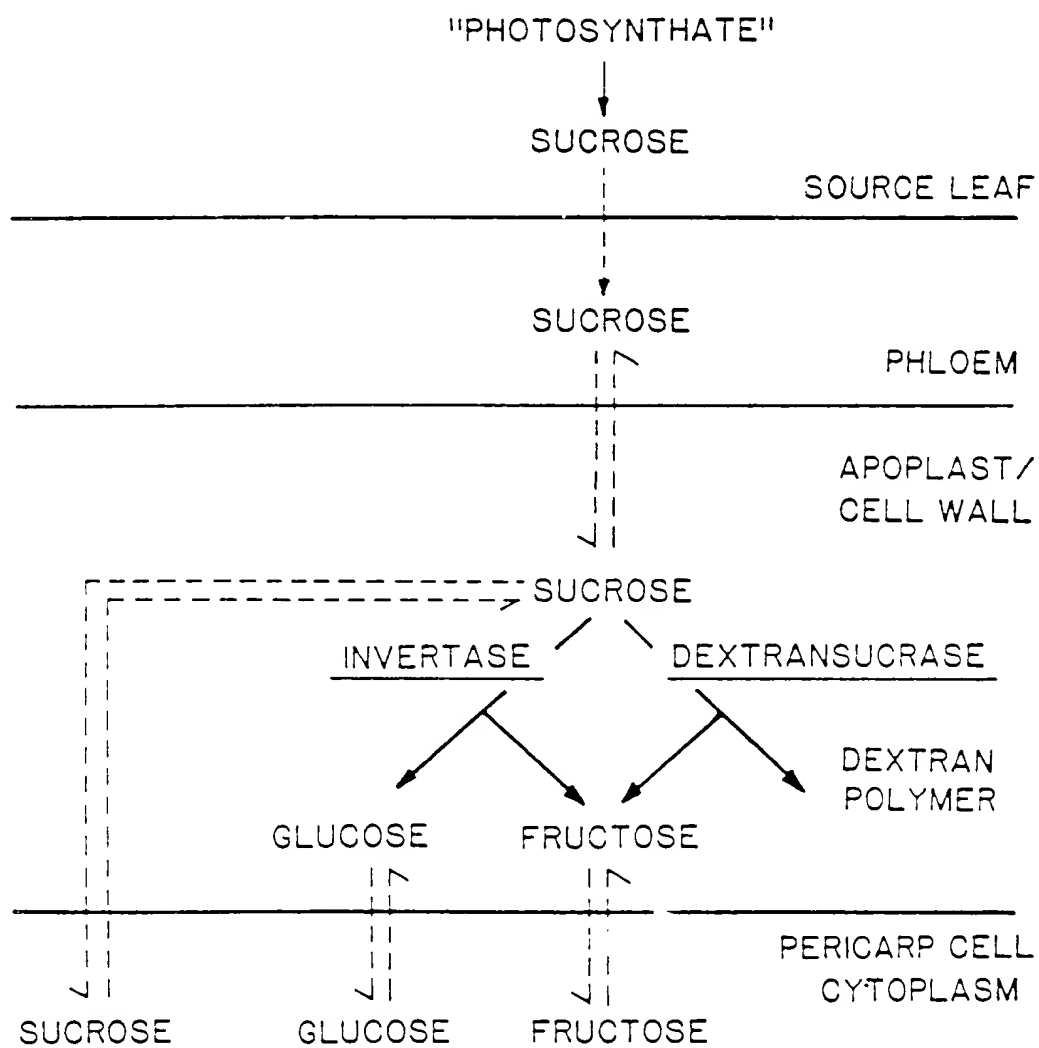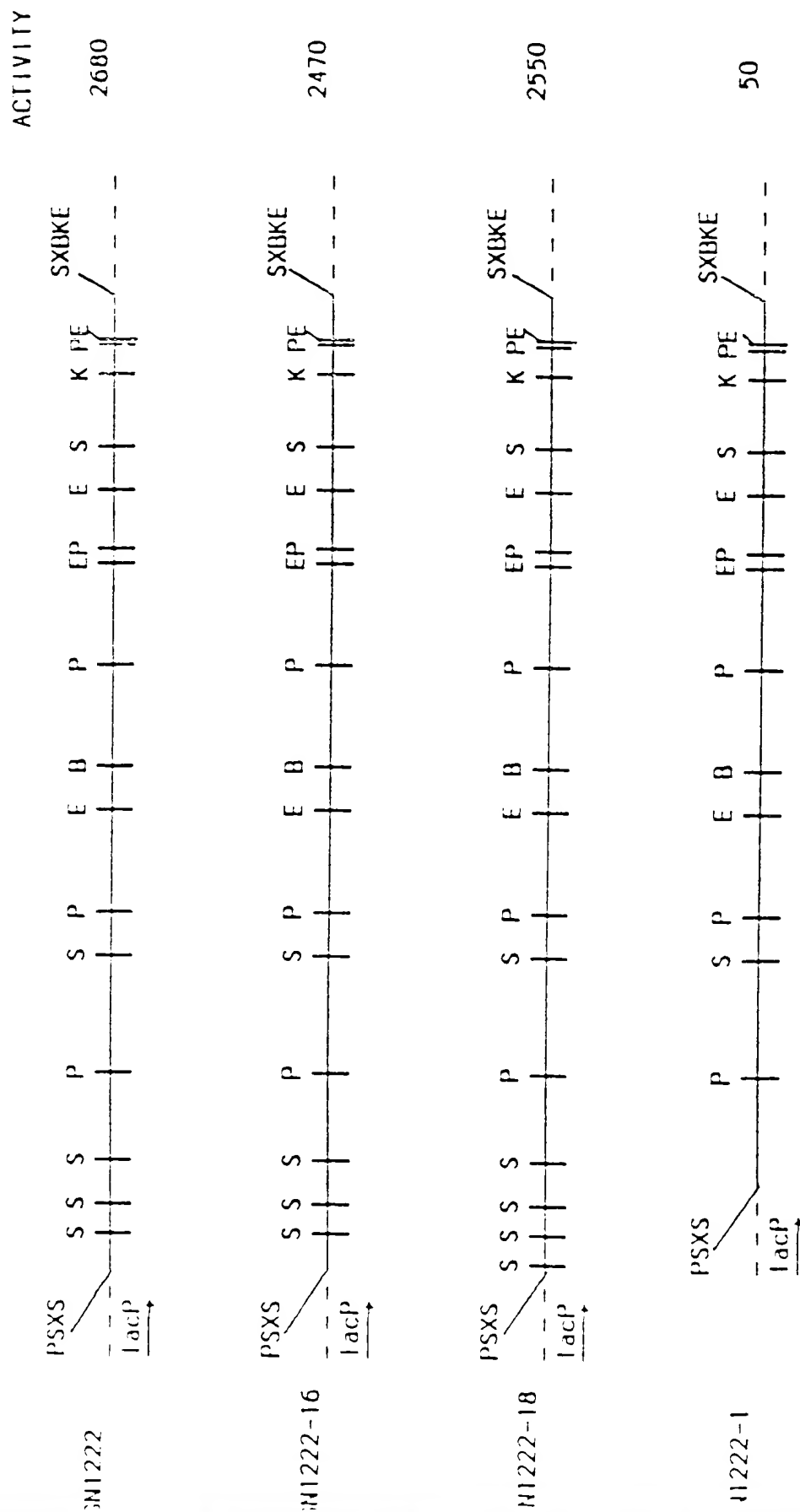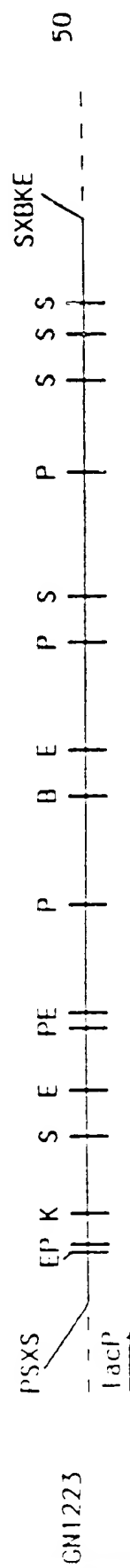
15

"PHOTOSYNTHATE"

↓

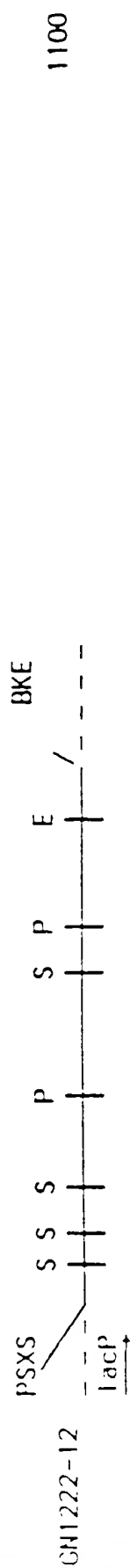SUCROSE

_____ SOURCE LEAF

SUCROSE

_____ PHLOEM

APOPLAST/
CELL WALL

SUCROSE

INVERTASE   DEXTRANSUCRASE

DEXTRAN
POLYMER

GLUCOSE   FRUCTOSE

_____ PERICARP CELL
CYTOPLASM

SUCROSE   GLUCOSE   FRUCTOSE

FIG. 1

FIG. 2-1

FIG. 2-2
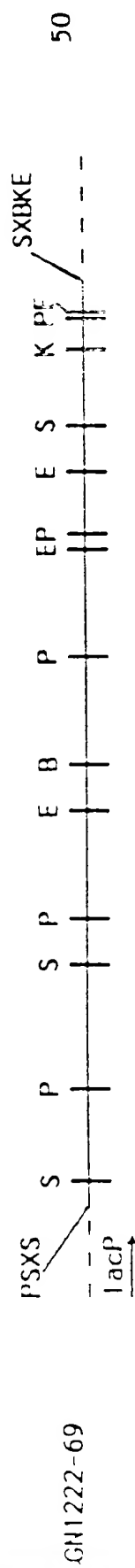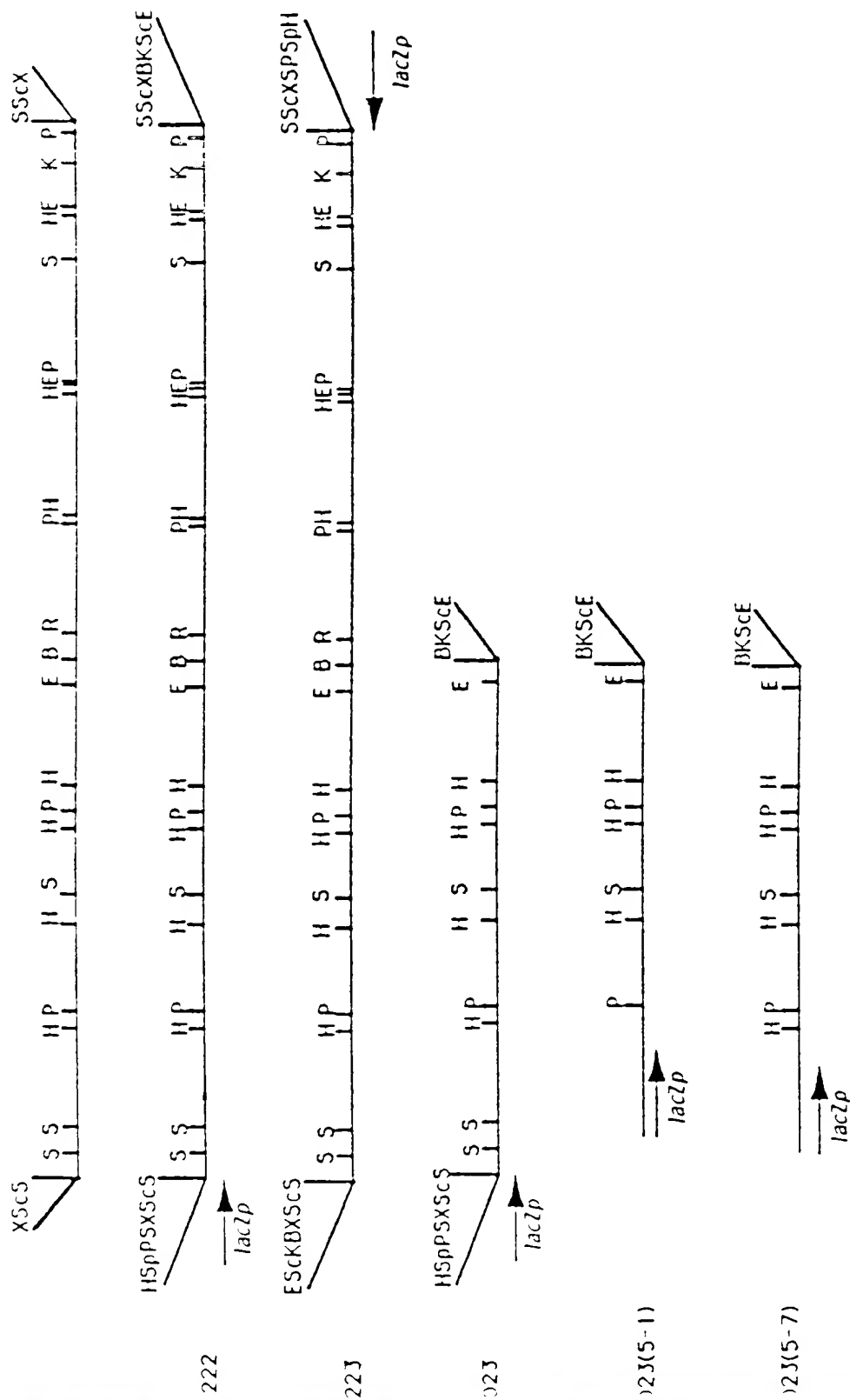
FIG. 3

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AATAAAATTAATGACTAATTAATAATATTAACATTGTTAACTTATCACTAGATAAGTAACAATGTTTT          71
                              ------------->
TTATAAAAAATTAATGTTTATATAATATTAATAATATAAATTCCTCGATTGACTGTTTTTACAATGTTTTTAAAA   142
ATATGTCGATTGTGTATTGAAATTTTTACTGTTTTTACAAATAAGTTATATAATTTTTCAATGTAATA          213
        -35                              <-------------
                                              -10

GAAGAGAATTATAAGGAGAAAATT ATG CCA TTT ACA GAA AAA GTA ATG CGG AAA AAG          273
          SD               M   P   F   T   E   K   V   M   R   K   K

CTT TAT AAA GTT GGG AAA AGT TGG GTA GTT GGT GTT TGT GCT TTT GCA TTA          327
 L   Y   K   V   G   K   S   W   V   V   G   V   C   A   F   A   L

ACC GCC TCA TTT GCT TTA GCA CCA AGT GTT TTA GGA GAC AGT AGT GTA CCT          381
 T   A   S   F   A   L   A   T   P   S   V   L   G   D   S   S   V   P

GAT GTG AGT GCG AAT AAC GTT CAA TCT GCT TCA GAT AAT ACA ACG GAT CAG          435
 D   V   S   A   N   N   V   Q   S   A   S   D   N   T   T   D   T   Q

CAG AAC ACT ACG GTT ACC GAA GAA AAT GAT AAA GTA CAG TCT GCA GCT AAT          489
 Q   N   T   T   V   T   E   E   N   D   K   V   Q   S   A   A   T   N

GAC AAT GTA ACA ACA GCT GCA AGC GAC ACA CAA TCT GCT GAT AAT AAT GTG          543
 D   N   V   T   T   A   A   S   D   T   T   Q   S   A   D   N   N   V

ACA GAA AAA CAG TCA TCA GAT GAT CAT GCA CTT GTT ACT GAA AAA GTC AAA          597
 T   E   K   Q   S   S   D   D   H   A   L   V   T   E   K   V   D   N   K

CAA GAT GAA GTC GCT CAA ACC AAT GTT ACT AGC AAA AAT GAG GAA TCA GCA GTT      651
 Q   D   E   V   A   Q   T   N   V   T   S   K   N   E   E   S   A   V

GCT TCA ACT GAC GAT CCT GCT GAA CCT GAT ACG ACA GAA ACA CAA CAA GTT          705
 A   S   T   D   T   D   P   A   E   T   D   E   T   Q   Q   V

AGC GGC AAG TAC GTT GAA AAA GAC GGT AGT TGG TAT TAT TTT GAT GAT GGC          759
 S   G   K   Y   V   E   K   D   G   S   W   Y   Y   F   D   D   G

AAA AAT GCT AAA GGT TTA TCA TCA ACG ATA GAC AAC AAT ATT CAA TTT TAC GAG      813
 K   N   A   K   G   L   S   T   I   D   N   N   I   Q   F   Y   E
```

FIG. 4-1

```
 867  AGT GGT AAA CAA GCC AAA GGA CAG TAT GTC ACA ATT GAT AAT CAA ACA TAT TAT
       S   G   K   Q   A   K   G   Q   Y   V   T   I   D   N   Q   T   Y   Y

 921  TTT GAT AAG GGC TCA GGT GAG TTA ACT GGT CTG CAA AGC ATT GAT CAA GGG AAC
       F   D   K   G   S   G   E   L   T   G   L   Q   S   I   D   Q   G   N

 975  ATA GTT GCT TTT AAC GAT GAA CAA CAA ATT TTT CAA ATT GAT AGC TAC GGG AAC
       I   V   A   F   N   D   E   Q   Q   I   F   Q   I   D   S   Y   G   N

1029  GAA AAT GGT ACA TAC TAC TTT GAT GAT AAA GGA CAC GCT GCT GGT ACC GGT ATT
       E   N   G   T   Y   Y   F   D   D   K   G   H   A   A   G   T   G   I

1083  AAG AAT ATC GAG GGC AAA AAT TAT TAT GAT CTT GGG CAA CTA AAA AAA GGG ATT
       K   N   I   E   G   K   N   Y   Y   D   L   G   Q   L   K   K   G   I

1137  GGC TTC TCT GGT GTG ATT GAT GGT CAA ATA ATG ACA TTT GAT CAG GAA ACA GGG
       G   F   S   G   V   I   D   G   Q   I   M   T   F   D   Q   E   T   G

1191  CAA GAA GTT TCT AAC ACT TCT GAA ATA TCT GCA CAT AAT TTG ACG ACT CAA AAC
       Q   E   V   S   N   T   S   E   I   S   A   H   N   L   T   T   Q   N

1245  ACG GAT TCT AAC AGT TAT AGC GCC CAC GGT ACG TTG GCT GAG GAC TTT GAA GAA
       T   D   S   N   S   Y   S   A   H   G   T   L   A   E   D   F   E   E

1299  AAT ATT GAC GGC TAT AGC GAA CAT AAT GCT CCA GGT ACA CTA ACT GAT CAA AAC
       N   I   D   G   Y   S   E   H   N   A   P   G   T   L   T   D   Q   N

1353  CGT AAC GGA ACA GAC TGG GAA CCT TCT CCA AAC ACC CAG GTC AAT GAC ATA TTG
       R   N   G   T   D   W   E   P   S   P   N   T   Q   V   N   D   I   L

1407  TCA GTG TGG TGG CCA GAT TGG GAA CCT TCT ACA GAT TTC AGA CCA ACT ATT TTA
       S   V   W   W   P   D   W   E   P   S   T   D   F   R   P   T   I   L

1461  GAT TTA GGG TTT ATC AGT AAT GCG GAC TCA AGT GAT AAT TTA AAT TAC ATG AGC
       D   L   G   F   I   S   N   A   D   S   S   D   N   L   N   Y   M   S

1515  TTA TTA AAT GAA GCA AGT AAT TAT GTT CAA AAG TCA ATT GAA ATG AAA ATT AGT
       L   L   N   E   A   S   N   Y   V   Q   K   S   I   E   M   K   I   S

1569  GCG CAA CCA CAG AGT ACA GAG TGG TTA AAG GAT GCA ATG GCA GAA ATT GTC GCG
       A   Q   P   Q   S   T   E   W   L   K   D   A   M   A   E   I   V   A

1623  CAA CCA CAG TGG AAT GAA ACT AGT GAA GAT ATG AGC AAT GAT CAT TTA CAA AAT
       Q   P   Q   W   N   E   T   S   E   D   M   S   N   D   H   L   Q   N

1677  GGC GCA TTA ACT TAT GTC AAC AGT GAC GCT CAT TTA CAA AAT TCA AAC TTT
       G   A   L   T   Y   V   N   S   D   A   H   L   Q   N   S   N   F
```

Fig. 4-2

```
AGA CTA CTT AAT CGG ACA CCA ACA AAC CAG ACT GGT GAA CAA GCC TAT AAT TTA   1731
 R   L   L   N   R   T   P   T   N   Q   T   G   E   Q   A   Y   N   L

GAT AAT TCA AAA GGT TTT GAA TTG TTA GCC AAT GAC AAT GTT GAT AAT TCA        1785
 D   N   S   K   G   F   E   L   L   A   N   D   N   V   D   N   S

AAC CCT GTA GTA CAA GCA GAA CAA TTG AAT TGG TTA TAT TAT TTA ATG AAT TTT    1839
 N   P   V   V   Q   A   E   Q   L   N   W   L   Y   Y   L   M   N   F

GGT ACG ATT ACG GCC AAC GAC GCG GAT GCT AAT TTT GAT GGT ATT CGT GTA GAT    1893
 G   T   I   T   A   N   D   A   D   A   N   F   D   G   I   R   V   D

GCA GTC GAC AAT GTG GAT GCT GAT TTG TTA CAA ATT GCT GCC GAT TAT TTC AAA    1947
 A   V   D   N   V   D   A   D   L   L   Q   I   A   A   D   Y   F   K

CTA GCT TAC GGT GTT GAT CAA AAT GAT GCT ACT CAG CAT CTT TCA ATT            2001
 L   A   Y   G   V   D   Q   N   D   A   T   N   Q   H   L   S   I

TTG GAA GAT TGG AGT CAC AAT GAT CCT TTG TAT GTA ACA GAT CAA GGA AGC AAT    2055
 L   E   D   W   S   H   N   D   P   L   Y   V   T   D   Q   G   S   N

CAA TTA ACC ATG GAT GAT TAT GTG CAC ACA CAA TTA ATC TGG TCT CTA ACA AAA    2109
 Q   L   T   M   D   D   Y   V   H   T   Q   L   I   W   S   L   T   K

TCA TCT GAC ATA CGA GGT ACA ATG CAG CGC TTC GTG GAT TAT ATG GTG GAT        2163
 S   S   D   I   R   G   T   M   Q   R   F   V   D   Y   M   V   D

CGA TCT AAT GAT AGT ACA GAA AAC GAA GCC ATT CCT AAT TAC AGC TTT GTA CGT    2217
 R   S   N   D   S   T   E   N   E   A   I   P   N   Y   S   F   V   R

GCA CAC GAC AGC GAA GTG CAA ACG GTT ATT GCC CAA ATT GTT TCC GAT TTG TAT    2271
 A   H   D   S   E   V   Q   T   V   I   A   Q   I   V   S   D   L   Y

CCT GAT GTT GAA AAT AGT TTA GCA CCA ACA ACA GAA CAA TTG GCA GCT GCT TTC    2325
 P   D   V   E   N   S   L   A   P   T   T   E   Q   L   A   A   A   F

AAA GTA TAC AAT GAA GAT GAA AAA TTA GCA GAC AAG AAG TAC ACA CAA TAT AAT    2379
 K   V   Y   N   E   D   E   K   L   A   D   K   K   Y   T   Q   Y   N

ATG GCT AGT GCT TAT GCG ATG TTG CTA ACC AAT AAG GAT ACT GTT CCT CGT GTC    2433
 M   A   S   A   Y   A   M   L   T   N   K   D   T   V   P   R   V

TAT TAT GGC GAT TTA TAT ACA GAT GAT GGT CAA TAT ATG GCA ACA AAG TCA CCA    2487
 Y   Y   G   D   L   Y   T   D   D   G   Q   Y   M   A   T   K   S   P
```

Fig. 4-3

```
TAC TAT GAT GCG ATT AAC ACT TTG CTA AAG GCT AGA GTT CAG TAT GTT GCT GGT   2541
 Y   Y   D   A   I   N   T   L   L   K   A   R   V   Q   Y   V   A   G

GGC CAA TCG ATG TCC GTT GAT AGT AAT GAC GTG TTA ACA AGT GTT CGC TAT GGT   2595
 G   Q   S   M   S   V   D   S   N   D   V   L   T   S   V   R   Y   G

AAA GAT GCC ATG ACA GCT TCT GAC AAC ACT GGA TCT GAG ACG CGT ACT GAA GGT   2649
 K   D   A   M   T   A   S   D   N   T   G   S   E   T   R   T   E   G

ATT GGA GTC ATC GTC AGC AAT AAC GCG GAG CTA CAA GAT GGG CAT GAG CAT ACT   2703
 I   G   V   I   V   S   N   N   A   E   L   Q   D   G   H   E   H   T

GTC ACA TTG CAT ATG GGG GCA GCT CAT AAG CAA AAC TAT CGT GCT TTG TTA TTA   2757
 V   T   L   H   M   G   A   A   H   K   Q   N   Y   R   A   L   L   L

TCA ACA ACT GCA GAT GCT AAC GGA TTA GCT TAT TAT GAT ACT GAA AAT GCA CCT GTG   2811
 S   T   T   A   D   A   N   G   L   A   Y   Y   D   T   E   N   A   P   V

GCG TAC ACA GAT GCT AAT GGA GAT CTA ATT TTT ACG TCA ATT TAT GGT   2865
 A   Y   T   D   A   N   G   D   L   I   F   T   S   I   Y   G

GTA CAA AAT CCA CAA GTT TCT GGT TAC TTG GCA GTT TGG GTT CCG GTA GGT GCG   2919
 V   Q   N   P   Q   V   S   G   Y   L   A   V   W   V   P   V   G   A

CAA CAA GAT CAA GAT GCC CGA ACG GAT ACA ACA ACA AAC ACG AGT GAT GAT   2973
 Q   Q   D   Q   D   A   R   T   D   T   T   T   N   T   S   D   D

AAA GTG TTC CAT TCA AAT GCA TTG TCA CAA GTC ATC TAC GAA GGT TTC   3027
 K   V   F   H   S   N   A   L   S   Q   V   I   Y   E   G   F

TCA AAC TTC CAA GCA TTT GCT ACA GAC AGC TAT ACA AAC GTA GTC ATC   3081
 S   N   F   Q   A   F   A   T   D   S   Y   T   N   V   V   I

GCT CAG AAT GAC CAA TTT AAG CAA TGG GGT GTG ACA AGC TTG GAT TCA ATT CAA AAC   3135
 A   Q   N   D   Q   F   K   Q   W   G   V   T   S   L   D   S   I   Q   N

CCA CAA TAT CGT TCA AGT GAT ACA TTG GAT CGT TAT ATT CAA AAC   3189
 P   Q   Y   R   S   S   D   T   L   D   R   Y   I   Q   N

GGG TAT GCA TTC ACG GAT TTA GGT TAT GAC CCG ACA AAA TAT   3243
 G   Y   A   F   T   D   L   G   Y   D   P   T   K   Y

GGA ACT GCT GAT CAG TTG CGC GAT GCT ATT AAA GCC TTA CAT GCT AGC GGT ATT   3297
 G   T   A   D   Q   L   R   D   A   I   K   A   L   H   A   S   G   I
```

FIG. 4-4

```
CAA GCC ATT GCC GAT TGG GTG CCG GAC CAA ATT TAT AAT TTG CCA GAG CAA GAA   3351
 Q   A   I   A   D   W   V   P   D   Q   I   Y   N   L   P   E   Q   E

TTA GCT ACT GTC ACA AGA ACA AAT TCA TTT GGA GAT GAC GAT ACT GAT TCT GAT   3405
 L   A   T   V   T   R   T   N   S   F   G   D   D   D   T   D   S   D

ATT GAC AAT GCC TTA TAT GTT GTA CAA AGT CGT GGT GGG TAT CAA TAT CAA GAG   3459
 I   D   N   A   L   Y   V   V   Q   S   R   G   G   Y   Q   Y   Q   E

ATG TAT GGT GGT GCC TTC TTA GAA TTA CAG GCA CTC CCA TAT TCC CTA TTT       3513
 M   Y   G   G   A   F   L   E   L   Q   A   L   P   Y   S   L   F

AAA GTG AAT CAA ATC TCA ACT GGC GTT CCA ATT GAT GGC AGT AAG ATT ACT       3567
 K   V   N   Q   I   S   T   G   V   P   I   D   G   S   K   I   T

GAG TGG GCG GCT AAG TAC TTC AAT GGC TCT AAC ATC CAA GGT GCT GGA G         3621
 E   W   A   A   K   Y   F   N   G   S   N   I   Q   G   A   G

TAC GTA TTG AAA GAT ATG GGT TCT AAT AAG TAC TTT AAG GTC TCG AAC ACT       3675
 Y   V   L   K   D   M   G   S   N   K   Y   F   K   V   S   N   T

GAG GAT GGT GAC TAC TTA CCA AAA CAG TTA ACT GAT CTG TCA GAA ACT GGC       3729
 E   D   G   D   Y   L   P   K   Q   L   T   N   D   L   S   E   T   G

TTT ACA CAC GAT GAT AAA GGA ATC ATC TAT TAT AGT GGT TAT CGT GCC           3783
 F   T   H   D   D   K   G   I   I   Y   Y   S   G   Y   R   A

CAA AAT GCA TTT ATT CAA GAT GAT AAT AAT TAT TAC TAT GAT TTT AAA ACA       3837
 Q   N   A   F   I   Q   D   D   N   N   Y   Y   Y   D   F   D   K   T

GGT CAT TTA ACA GGT TTG CAA AAG ATT AAT AAC CAT TAC TTC TTC TTA           3891
 G   H   L   T   G   L   Q   K   I   N   N   H   Y   F   F   L

CCT AAT GGT ATC GAA CTG GTC AAG AGC TTA CAA GAT GGT ACA ATT               3945
 P   N   G   I   E   L   V   K   S   L   Q   N   E   D   G   T   I

GTT TAT TTC GAT AAG AGT CAT TTT GAT GCT TAT ATA ACT GAT CAA               3999
 V   Y   F   D   K   S   H   Q   V   F   D   A   Y   I   T   D   Q

AAT GGA AAT GAT GGT GTA ATG CTT AAA TCA GAT CAA CTT                       4053
 N   G   N   A   Y   F   D   V   M   L   K   S   G   L

GCA ACG ATT GAT GGA CAT TTT GAT CAA TAT GGT GTC CAG GTT AAG               4107
 A   T   I   D   G   H   F   D   Q   Y   G   V   Q   V   K
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G.4-5

```
GAT AAG TTT GTG ATT GGC ACT GAT GGT TAT AAG TAT TAC TTT GAA CCA GGT AGT   4161
 D   K   F   V   I   G   T   D   G   Y   K   Y   Y   F   E   P   G   S

GGT AAC TTA GCT ATC CTA CGT TAT GTG CAA AAT AGT AAG CAA TGG TTC TAT       4215
 G   N   L   A   I   L   R   Y   V   Q   N   S   K   Q   W   F   Y

TTT GAT GGT AAT GGC CAT GCT GTC ACT GGT TTC CAA ACA ATT AAT GGT AAA AAA   4269
 F   D   G   N   G   H   A   V   T   G   F   Q   T   I   N   G   K   K

CAA TAT TTC TAT AAT GAT GGT CAT CAA AGT GGT GAA TTC GAT GCA GAC           4323
 Q   Y   F   Y   N   D   G   H   Q   S   G   E   F   D   A   D

GGG GAT ACT TTC TAT ACG AGT GCC ACT GAT GGT CGC CTA GGT GTT CAG           4377
 G   D   T   F   Y   T   S   A   T   D   G   R   L   G   V   Q

AAG ATT AAT GGT ATT ACC TAT GCT TTT TAT AAC ACA GGA AAT ATC ACA AAT       4431
 K   I   N   G   I   T   Y   A   F   Y   N   T   G   N   L   T   N

CAG TAT TAT CAA TTA GCA GAT GGT AAA TAT ATG TTG TTA GAT AGT GGT CGT       4485
 Q   Y   Y   Q   L   A   D   G   K   Y   M   L   L   D   S   G   R

GCG AAA ACA GGG TTT GTA TTG CAA GAT GGT GTA CTA GAT TTC GAT CAA AAC       4539
 A   K   T   G   F   V   L   Q   D   G   V   L   R   Y   F   D   Q   N

GGT GAG CAA GTG AAA GAT GCT ATC ATT GTG GAT CCA GAT ACT AAC TTG AGT TAT   4593
 G   E   Q   V   K   D   A   I   I   V   D   P   D   T   N   L   S   Y

TAT TTC AAT GCA ACA CAA GGT GTC GTA GCT AAA TAT CAA CTT TTC GAG TAT CAA   4647
 Y   F   N   A   T   Q   G   V   A   V   K   N   D   Y   F   E   Y   Q

GGT AAT TGG TAT TTA ACA GAT GCT AAT TAT TTT GAT GAA GTT TTT AAA GCA GAT   4701
 G   N   W   Y   L   T   D   A   N   Y   Q   L   I   K   G   F   K   A

GTT GAC GAC AGC TTA CAA CAT TTT GAT GAA GTC ACT GGT GTA CAA ACA AAA GAT   4755
 V   D   D   S   L   Q   H   F   D   E   V   T   G   V   Q   T   K   D

AGT GCT TTA ATA AGT GCT CAG GGT AAG GTT TAC CAA TTT GAT AAT GGA AAT       4809
 S   A   L   I   S   A   Q   G   K   V   Y   Q   F   D   N   G   N

GCT GTG TCA GCA TAA GCTTTCTGTATATATAGTGAAAGCCAAGGTCCTTTGACCTTTGGCTTT      4875
 A   V   S   A  END                            ------------>   <--------

TTGATTTAATTGTCTATTGAATTCCTTGATGATATCGGCTAATTAATTTATTTATTCATTCCTTTTTCGGC  4946
```

G.4-6

AMINO ACID COMPOSITION – DEXTRANSUCRASE

FIG. 5

FIG. 6

FIG. 7

Insert from original dex clone pCGN1222:

P = PstI
S = SalI
H = HindIII
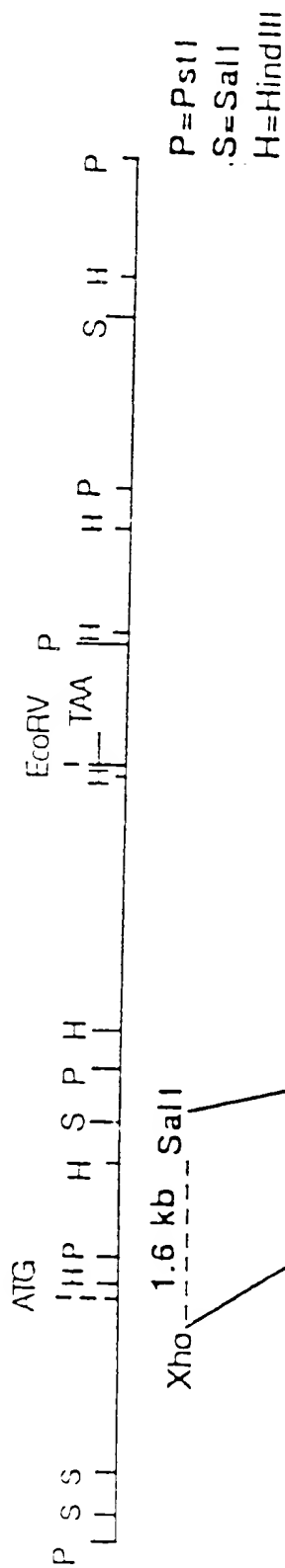
Generate 1.6kb 5' end of gene as PCR fragment using primers Dex02 and Dex03. Clone fragment as XhoI/SalI fragment into pCGN2303 (Bluescript vector with 3.0 kb 3' end of gene).
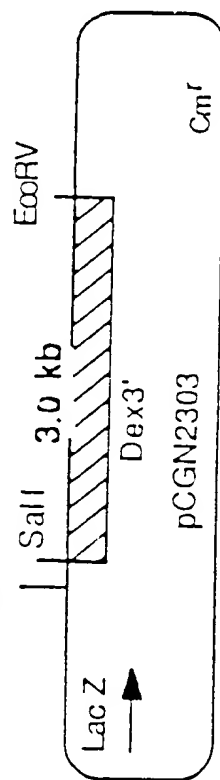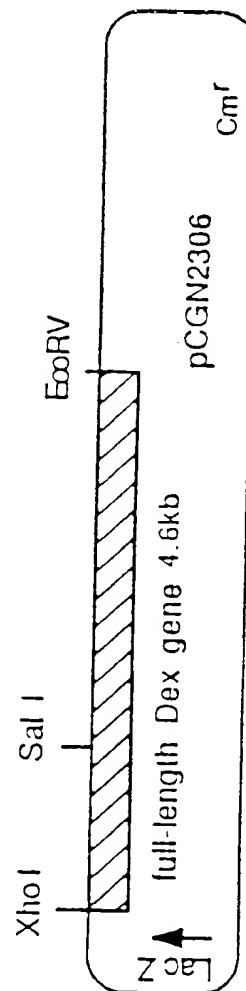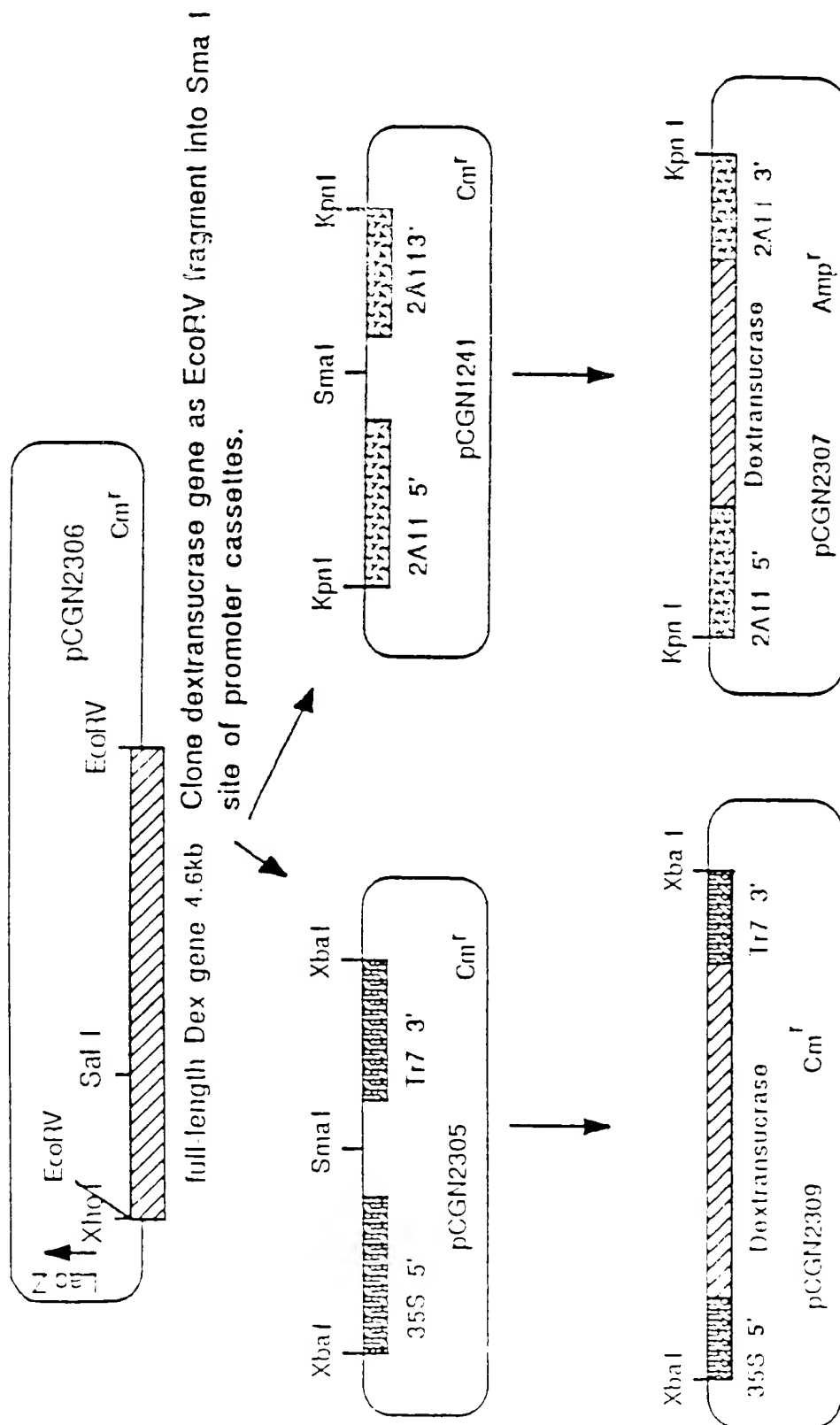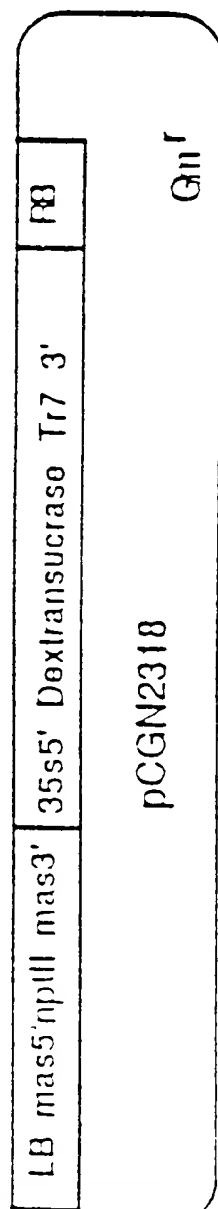
FIG. 8

FIG. 9-1

pCGN2306 Cm^r

EcoRV EcoRV

Sal I

Xho I

full-length Dex gene 4.6kb   Clone dextransucrase gene as EcoRV fragment into Sma 1 site of promoter cassettes.

Kpn1 Sma1 Kpn1

2A11 5'   2A11 3'

pCGN1241   Cm^r

Xbal Sma1 Xbal

35S 5'   Tr7 3'

pCGN2305   Cm^r

Kpn I Kpn I

2A11 5'   Dextransucrase 2A11 3'

pCGN2307   Amp^r

Clone promoter + dextransucrase gene into binary vector pCGN1547 as Xba1 fragment (for 35S) and Kpn1 fragment (for 2A11).

Xbal Xba I

35S 5'   Dextransucrase Tr7 3'

pCGN2309   Cm^r
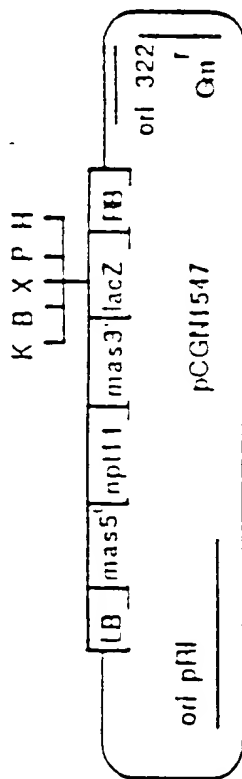
pCGN2319 = LB mas5' nptIII mas3' Tr7 3' Dextransucrase 35S5' RB
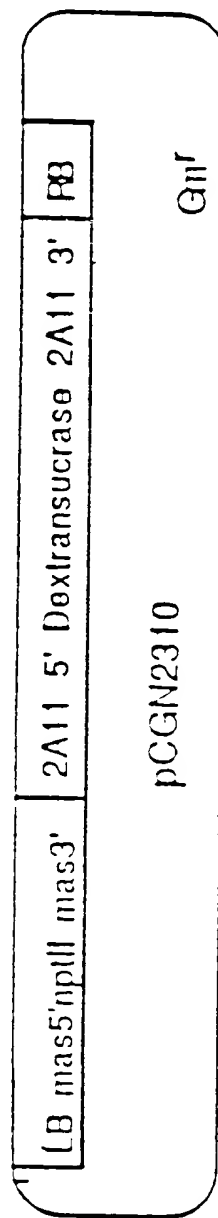
pCGN2311 = LB mas5' nptIII mas3' 2A11 3' Dextransucrase 2A11 5' RB

FIG. 9-2

Insert from original dex clone pCGN1222:

Generate 1.6kb 5' end of gene as PCR fragment -
clone as XhoI/SalI fragment into pCGN2303

(Bluescript vector with 3.0 kb 3' end of dex gene)

P=PstI
S=SalI
H=HindIII
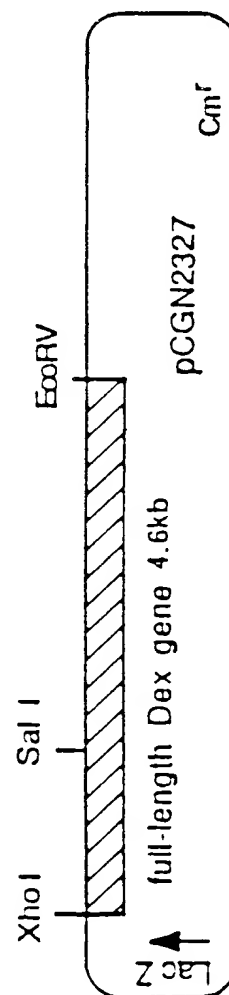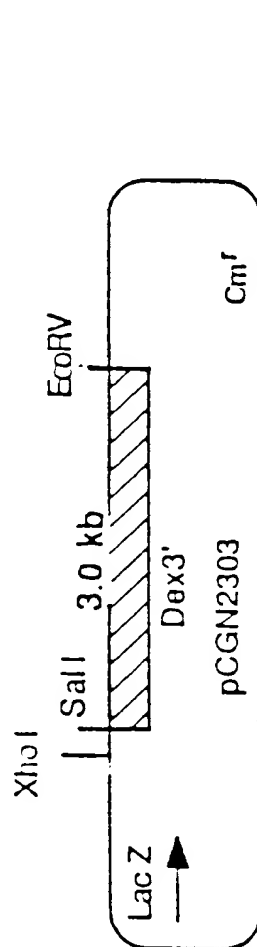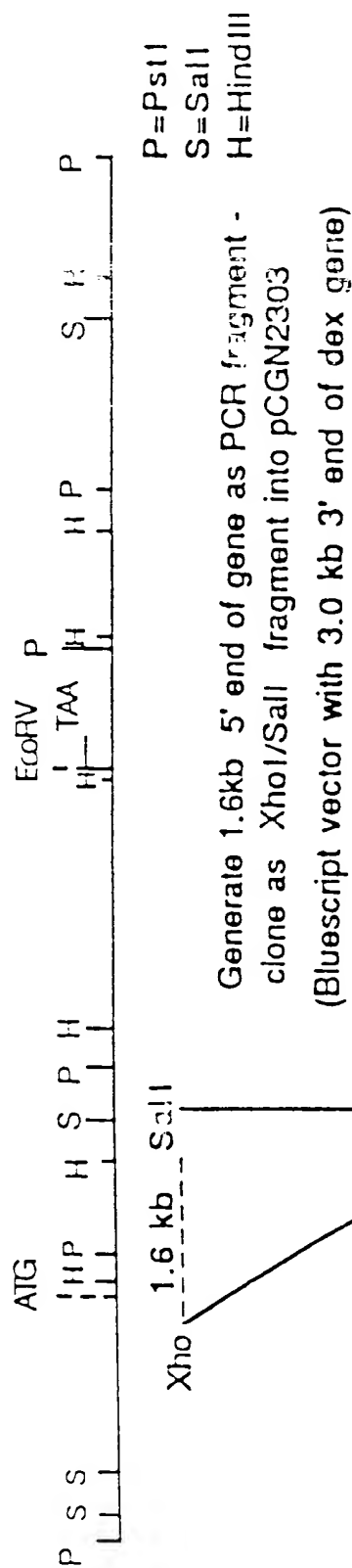
FIG. 10

FIG. 11-1

PG leader and 5' region from pCGN1407:
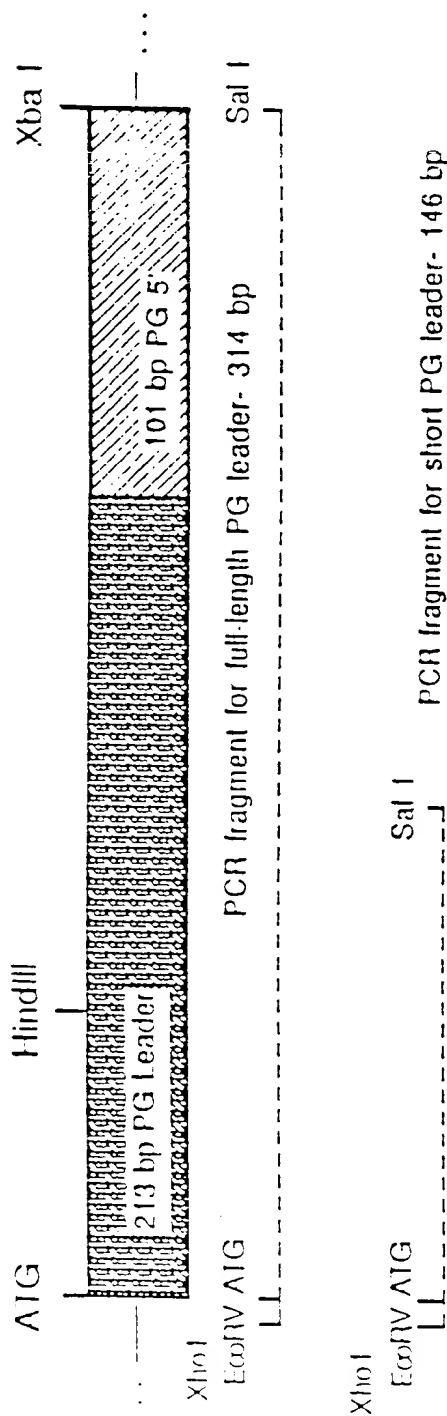
PCR fragments have XhoI, EcoRV, and SalI sites for cloning

Full-Length PG Leader/Dextransucrase

XhoI EcoRV
SalI
EcoRV
pCGN2330
cm^r
LacZ
Dextransucrase Gene

Short Version PG Leader/Dextransucrase

XhoI EcoRV
SalI
EcoRV
pCGN2331
cm^r
LacZ
Dextransucrase Gene

FIG. 11-2

FIG. 12A

FIG. 12B

INTERNATIONAL SEARCH REPORT

International Application No PCT/US89/02729

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): A01H 1/04; C12N 1/00; C12N 5/00; C12N 15/00; C07H 21/04
U.S.CL.: 800/1; 435/172.1, 172.3, 240.4, 317.1, 320; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S.	536/27 435/172.1, 172.3, 240.4, 317.1, 320 800/1

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

USPTO Automated Patent System; DIALOG files: BIOTECH, PATENTS, WORLD PATENTS INDEX, See Attachment for search terms.

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
P,X,Y	US, A, 4,801,540 (HIATT) 31 January 1989, See entire document and page 4, lines 65-68.	1-30
A	Hortscience (St. Joseph, Michigan USA), Volume 13, Issued December 1978, Stevens et al, "Genetic potential for overcoming physiological limitations an adaptability, yield, and quality in the tomato", pages 673-678.	1-30
Y	Journal of Bacteriology (Washington DC, USA) Volume 153, Issued 1983, Gay et al., "Cloning structural Gene sacB, which codes for exenzyme levansucrase of Bacillus subtilis: Expression of the gene in E. coli", pages 1424-1431.	1-30

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
September 1989	30 OCT 1989

International Searching Authority	Signature of Authorized Officer
ISA/US	P. Rhodes P. Rhodes

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	Theoretical and Applied Genetics (New York, USA) Volume 73, Issued 1987, Osborn et al, "Identification of restriction fragment length polymorphisms linked to genes controlling soluble solids content in tomato fruit", pages 350-356.	1-30

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers   because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

   SEE ATTACHMENT.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

   Telephone Practice

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.